University of Alberta

Actions of estrogen in the hypothalamus: signalling through estrogen receptor beta and nitric oxide

by

Sarah Gingerich

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Abstract

Estrogen is an important signalling molecule in the reproductive, cardiovascular, skeletal, and central nervous systems. Our laboratory previously showed that 17β-estradiol (E2) acts in the brain on the gaseous neurotransmitter, nitric oxide (NO), to attenuate blood pressure responses to restraint stress in ovariectomized rats. The purpose of this thesis is to demonstrate that E2 acts in the paraventricular nucleus (PVN) of the hypothalamus to modulate the NO system and to identify the molecular mechanisms through which E2 modulates the NO system in neurons.

We showed that E2 alters the expression of endothelial nitric oxide synthase (eNOS) and neuronal NOS (nNOS) exclusively in the PVN of rat hypothalamic slice cultures, but not in surrounding hypothalamic regions, suggesting that the PVN is a specific target of E2 action. By using pharmacological agents selective for ERα and ERβ, we demonstrated that ERβ mediates the effects of E2 on NOS expression in the PVN.

We investigated the physiological effects of E2 in the PVN on autonomic function by determining the effects of E2 microinjection into the PVN of anesthetized male rats on mean arterial pressure and heart rate. We showed E2 microinjected into the PVN attenuates the L-glutamate-induced pressor response. We also determined that ERβ and NO produced by eNOS and nNOS mediate the effects of E2 in the PVN on the L-glutamate-induced pressor response.

Finally, we identified the molecular mechanisms involved in stimulating the NO system by ERβ activation in neurons of the hypothalamus. We found that activation of ERβ in rat primary hypothalamic neurons leads to an increase in NO production by increasing NOS activity through its phosphorylation at Ser^{1412}. We further determined

that the Src/PI3K/Akt pathway mediates the effects of ERβ activation on levels of nNOS phosphorylation and NO production.

The findings described in this thesis demonstrate that E2 signals through ERβ and NO in the PVN to modulate autonomic function and characterize the signal transduction pathway leading to the stimulation of NO by ERβ in neurons. Thus, these findings contribute to our understanding of how E2 signals in neurons and the physiological outcomes of E2 action in the brain.

Dedicated to my husband Ryan

Every step I have taken on this journey has been with you by my side

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LIST OF ABBREVIATIONS

7-NiNa	7-nitroindazole sodium salt
ACTH	adrenocorticotropin hormone
AF-1	activation function domain-1
AF-2	activation function domain-2
ANOVA	analysis of variance
AP-1	activator protein-1
AUC	area under the curve
BME	basal medium eagle
BP	blood pressure
BSA	bovine serum albumin
Ca^{++}	Calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREs	cAMP response elements
CREB	cAMP reponse element binding protein
CRH	corticotropin releasing hormone
DAB	3,3'diaminodenzidine tetrahydrochloride
DAN	2,3 diaminonaphthalene
DAPI	3',6-diamindino-2-phenylindole
DBD	DNA-binding domain
DIV	days *in vitro*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
E2	17β-estradiol, estrogen
ER	estrogen receptor
ERE	estrogen response element
eNOS	endothelial nitric oxide synthase
FSH	follicle stimulating hormone
GABA	γ-animobutyric acid
GFAP	glial fibrillary acidic protein
GnRH	gonadotropin-releasing hormone
GPR30	G protein-coupled receptor 30
HBSS	hank's balanced salt solution
HPA	hypothalamic-pituitary-adrenal axis
HPG	hypothalamic-pituitary-gonadal axis
HR	heart rate
HRT	hormone replacement therapy
HSP 90	heat-shock protein 90

I.C.V	intracerebroventricular
iNOS	inducible nitric oxide synthase
LBD	ligand-binding domain
LH	luteinizing hormone
L-NIO	N5-(10Iminoethyl)-L-ornithine
LTP	long-term potentiation
NADPH	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartic acid
MAP-2	microtubule-associated protein-2
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MNAR	modulator of non-genomic activity of estrogen receptor
MPP	methyl-piperidino-pyrazole
mRNA	messenger RNA
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
L-NAME	N-nitro-L-arginine methyl ester
LPS	lipopolysaccharide
nNOS	neuronal nitric oxide synthase
NTS	nucleus of the solitary tract
OVX	ovariectomy
PI3K	phosphatidylinositol-3 kinase
PBN	parabrachial nucleus
PBS	phosphate buffered saline
PFA	paraformaldehyde
PPT	propyl-pyrazole-triol
PVN	paraventricular nucleus
RECA-1	rat endothelial cell marker
RNA	ribonucleic acid
R,R-THC	R,R-tetrahydochrysene
RVLM	rostral ventral lateral medulla
SCM	serum-containing medium
SFM	serum-free medium
siRNA	small interfering RNA
SON	supraoptic nucleus
Src	steroid receptor co-activator
TE	tris-EDTA
VMH	ventral medial hypothalamus
WHI	women's health initiative

Chapter 1

Introduction

1.1 Estrogen's discovery and synthesis

Estrogen was discovered in 1923 by two American scientists, Edgar Allen and Edward Doisy, as the primary female sex hormone responsible for regulating female reproduction [1]. Circulating estrogen takes three forms, the most abundant being 17β-estradiol (E2) followed by estriol and estrone. E2 is primarily synthesized in granulosa cells of the ovaries [2], where cholesterol is metabolized into testosterone which is further reduced by the enzyme aromatase into E2 (Figure 1-1a). Levels of circulating E2 are regulated by levels of the gonadotrophs, follicle stimulating hormone (FSH) and luteinizing hormone (LH), released from the anterior pituitary gland [3].

Since its discovery over 80 years ago the understanding of estrogen's role in human physiology has expanded beyond its role in female reproduction. Estrogen is now appreciated as a key player in many physiological systems including the cardiovascular, skeletal and central nervous systems.

1.2 Physiological roles of estrogen

1.2.1 Reproductive system

Female reproductive function is coordinated by the reproductive cycle, known as the estrus cycle in rats and the menstrual cycle in humans. The estrus cycle is composed of four phases; proestrus, estrus, metestrus, and diestrus. Levels of circulating E2 are high during proestrus and low during diestrus [4]. E2 plays an important role in regulating female reproduction through its actions on the hypothalamic-pituitary-gonadal (HPG) axis [5]. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) from the paraventricular nucleus into the anterior pituitary gland, via the hypophyseal portal system of the median eminence, where GnRH stimulates the secretion of FSH and LH into the circulation. FSH and LH stimulate the synthesis and release of E2 from the ovaries [3]. Circulating E2 then negatively feeds back on GnRH-producing neurons of the paraventricular nucleus to inhibit GnRH-induced LH release. During proestrus, E2 positively feeds back on GnRH neurons to induce a pre-ovulatory rise in LH release [5, 6]. Beside its role in the HPG axis on GnRH secretion, E2 also regulates vascularization of the endometrium in females [7]. Estrogen is also essential to the reproductive success of both sexes, as studies from knockout mice revealed that both male and female mice lacking estrogen receptors are infertile [2]. In males, E2 plays a important role in the development of the prostate gland [8] and testis [9, 10].

1.2.2 Skeletal system

E2 plays an important role in regulating skeletal health in men and women by inhibiting bone turnover through the inhibition of bone resorption and enhancement of bone formation [11, 12]. In women, estrogen deficiency is a risk factor for osteoporosis [12, 13]. E2 also protects against bone loss, as postmenopausal women receiving E2 therapy have higher bone mineral densities and fewer fractures compared to postmenopausal women not receiving therapy [14]. Furthermore, in female rats E2 attenuates the increase in bone turnover induced by ovariectomy [15, 16].

1.2.3 Cardiovascular system

The loss of estrogen in postmenopausal women greatly increases the risk of cardiovascular disease [17], which is the leading cause of death amongst these women [17, 18]. E2 plays a crucial role in cardiovascular health through its direct and indirect actions on the cardiovascular system. E2 treatment induces vasodilation of the femoral and isolated mammary arteries of ovariectomized (OVX) mice [19] and of men [20], respectively, through its actions on the vasoactive molecule, nitric oxide. E2 also stimulates cell proliferation [21, 22] and migration [22, 23] of cultured endothelial cells. Several studies suggest that E2 provides cardioprotection by attenuating key events in the pathogenesis of cardiovascular diseases such as atherosclerosis. These events include the proliferation/migration of smooth muscle cells and inflammation[24]. Specifically, E2 attenuates the proliferation of rat aortic smooth muscle cells induced by fetal calf serum[25] and the migration of smooth muscle cells induced by chemoattractants [26]. Furthermore, E2 attenuates the expression of the inflammatory enzyme, cyclooxygenase-2, in cerebral vessels of OVX rats induced by lipopolysaccharide [27] and interleukin-1β [28].

1.2.4 Central nervous system

E2 plays a critical role in both the developing and adult central nervous systems (CNS) as it influences the development, survival, plasticity, and aging of neurons. During development, E2 plays a key role in sexual differentiation of the brain. The onset of sexual differentiation appears to primarily occur during a brief perinatal period, where the greatest differences in E2 synthesis between sexes are observed [29, 30]. One of the best characterized morphological sexual dimorphism in the brain is in the medial preoptic area. In male rats this area is over 3-fold greater in volume compared to female rats [31, 32]. E2 synthesized through the aromatization of testosterone mediates the masculinization of this region [32] and E2 treatment in neonatal female rats masculinizes the medial preoptic area by increasing its volume over 3-fold [31, 32]. Functional sexual dimorphism is also observed in the hypothalamic-pituitary-adrenal axis, as basal and stress-induced levels of adrenocorticotropin hormone and corticostrone are higher in females compared to males [33-35]. McCormick et al. demonstrated that the stress-induced increase in corticosterone levels of adult male rats that were gonadectomized and treated neonatally with E2 are attenuated compared to those treated neonatally with vehicle [34].

The actions of E2 in the brain also regulate reproductive behaviours such as lordosis, which allows for copulation in the female rat. E2 has been demonstrated to regulate lordosis, as E2 treatment facilitates lordosis behavior in OVX rats [36] and ERα knockout mice fail to exhibit lordosis behavior [37]. Recent studies have suggested that E2 influences lordosis by increasing spine density and neuronal activity in the ventral medial hypothalamus (VMH) of OVX rats [38, 39]. In concert with the effects of E2 on neuronal activity of the VMH, E2 induces ovulation through its actions on GnRH, synchronizing ovulation with lordosis behavior [40].

E2 also enhances neurite growth of hypothalamic explant cultures [41] and hypothalamic primary neurons [42]. During development E2 influences the expression of neurotransmitters and neuropeptides [29, 43]. E2 has been shown to enhance spatial [44] and working memory [45] in OVX rats. E2 is suggested to influence memory via its actions on synaptic plasticity [39], such as increased dendritic spine density in CA1 pyramidal neurons of the hippocampus induced by E2 [46, 47].

Clinical studies and *in vivo* models of neurodegeneration and neuronal injury have demonstrated that E2 plays an important protective role against neurodegenerative diseases and brain injury. E2 treatment in postmenopausal women appears to be protective against stroke [48] and Alzheimer's disease [49, 50]. In ischemia models, E2-

treated OVX rats have a reduced infarct volume compared to untreated OVX rats [51, 52]. In a murine model of Parkinson's disease, neurotoxicity is attenuated in OVX rats receiving E2 treatment, as determined by smaller decreases in levels of dopamine and its metabolites [53]. E2 also protects against brain injury in OVX rats via anti-apoptotic mechanisms through the up regulation of pro-survival genes, such as bcl-2 [51].

In vitro studies have demonstrated that E2 protects cortical neurons from glutamate toxicity through a phosphatidyl-inositol-3 kinase (PI3K) pathway [54] and a mitogen-activated-protein kinase (MAPK) pathway [55]. The MAPK pathway also mediates the neuroprotective effects of E2 against oxidative stress in a hippocampal cell line, HT22 [56]. In hippocampal neurons E2 protects against glutamate-induced neurotoxicity by increasing the amount of cytosolic calcium sequestrated by the mitochondria, an event that is mediated by the up-regulation of bcl-2 in the mitochondria [57]. Furthermore, it was recently suggested that the molecular mechanism through which E2 increases bcl-2 expression is initiated by the influx of calcium through L-type calcium channels leading to the activation of a Src/ERK/CREB pathway [58].

1.2.4.1 Autonomic nervous system

E2 acts in the hypothalamus to regulate homeostatic mechanisms [59]. A well characterized role of E2 in the hypothalamus is its role in the hypothalamic-pituitary-gonadal axis, as described in section 1.2.1. E2 also regulates feeding, as feeding is increased in OVX rats, while food intake is decreased in OVX-E2 treated rats [60]. Hot flashes, caused by sudden changes in the hypothalamic control of temperature, are a common symptom of menopause [61]. E2 therapy decreases the number of hot flashes in ostmenopausal women [61, 62], due in part to an increase in the sweating threshold [62].

Estrogen is an important regulator of autonomic function as the loss of estrogen increases sympathetic activity [63-65] and dampens baroreflex sensitivity [63, 66]. Postmenopausal women receiving E2 replacement therapy display decreased sympathetic activity [63, 67, 68] and increased baroreflex sensitivity [69, 70] compared to those not receiving E2 therapy. Resting blood pressure is also lower in pre-menopausal women compared to postmenopausal women and age-matched men [63, 71]. In rat models of hypertension, including spontaneously hypertensive rats [72], New Zealand genetically hypertensive rats [73], and Dahl-salt sensitive rats [74], resting blood pressure is lower in females compared to males. Peripherally administered E2 decreases heart rate, renal and splanchnic sympathetic nerve activity, and attenuates the phenylephrine-induced pressor response in conscious OVX rats [75]. Similarly, in anesthetized OVX rats peripheral injection of E2 decreases blood pressure, heart rate, renal sympathetic nerve activity, and attenuates the phenylephrine-induced pressor response [76]. E2 administered peripherally to anesthetized OVX rats enhances the depressor effect induced by electrical stimulation of the aortic depressor nerve and is thought to act through a centrally mediated pathway [77].

E2 in the brain has been shown to modulate blood pressure and sympathetic output. Bilateral injections of E2 into autonomic centers of the brain, such as the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM), have been shown to decrease resting blood pressure, decrease renal sympathetic nerve activity and enhance baroreflex sensitivity to phenylephrine in male [78], OVX [79], and OVX-E2 [79] treated rats.

E2 also plays an important role in regulating the function of the autonomic and neuroendocrine systems during the homeostatic response to stress. E2 decreases the number of activated neurons in autonomic/neuroendocrine nuclei of OVX rats following foot shock- [80], immobilization- [81], and noise-induced stress [82]. Blood pressure responses to mental stress are attenuated in postmenopausal [83, 84] and peri-menopausal [85] women receiving E2 replacement therapy compared to those receiving those not receiving E2 therapy. We and others have shown that blood pressure responses to restraint stress [86-88] and cage switch stress [87] in OVX rats treated with E2 are attenuated compared to vehicle-treated OVX rats.

The hypothalamic-pituitary-adrenal (HPA) axis plays a major role in the neuroendocrine response to stress. Stress induces the release of corticotropin-releasing-hormone (CRH) and vasopressin from the paraventricular nucleus of the hypothalamus into the anterior pituitary gland, via the hypophyseal portal system of the median eminence, inducing the release of adrenocorticotropin (ACTH) hormone into the circulation. ACTH induces the release of corticosterone from the adrenal glands to modulate physiological functions in response to the stress. Corticosterone also acts through a negative feedback loop to slow the release of CRH from the paraventricular nucleus [33]. The literature on the effects of E2 on the HPA stress response is conflicting. Some groups demonstrate that E2 potentiates the stress-induced increase in ACTH levels in OVX rats [89], while other studies show that E2 attenuates the increase [82, 88]. In addition, E2 treatment in peri-menopausal women has been shown to attenuate glucocorticoid and catecholamine release in response to mental stress [85]. Although the reasons for these divergent findings are unclear at this time, a recent study by Lund *et al.*

suggests that the effect of E2 on HPA function during stress depends on the specific type estrogen receptor activated [90]. Further studies are needed to clearly define the mechanisms through which E2 modulates the HPA axis in response to stress.

1.3 Estrogen receptors

1.3.1 Types of receptors

Over the past 20 years our knowledge of estrogen receptors (ERs) and their function has evolved from the idea that a single nuclear ER carries out all of estrogen's effects to our current understanding that the effects of estrogen are orchestrated by a variety of nuclear- and membrane-bound ERs. The first ER discovered in 1986 was ERα and was cloned from the breast cancer cell line MCF-7 [91, 92]. For an entire decade ERα was believed to be the only ER. However, in 1996 a second ER, ERβ, was discovered and cloned from the prostate gland of rat [93]. ERα and ERβ belong to a superfamily of highly conserved nuclear receptors that function as transcription factors [94, 95]. The distribution patterns of ERα and ERβ in the body have been examined and, while some tissues express both ERs, others exclusively express either ERα or ERβ. For example, the uterus, ovary, and brain express both ERs, the adrenal gland and kidney exclusively express ERα, and the bladder, and lung exclusively express ERβ [96].

In 2004 a third ER was identified, G protein-coupled receptor 30 (GPR30), a G protein-coupled receptor that carries out rapid membrane initiated actions of estrogen [97]. GPR30 mRNA is expressed in several tissues including ovary, lung and prostate [98]; more recently GPR30 protein was found to be expressed in many regions of the brain [99].

1.3.2 Distribution of ERs in the CNS

ERs are found to some extent in virtually every region of the brain and spinal cord [100]. ERβ has been identified as the predominant ER in the CNS [100]. Brain regions including the olfactory tubercule, bed nucleus of the stria terminalis, and nucleus of the solitary tract (NTS) express both ERα and ERβ equally [100]. ERα is exclusively expressed in the ventral medial hypothalamus and the subfornical organ [100]. ERβ is exclusively expressed in various nuclei including the supraoptic nucleus (SON), paraventricular nucleus (PVN), and suprachiasmatic nucleus [100]. The cortex and hippocampus predominantly express ERβ although lower levels of ERα are also expressed here [100, 101]. Earlier this year the distribution pattern of GPR30 in the CNS was investigated. GPR30 was found to be expressed in many nuclei including the PVN, SON, arcuate nucleus, hippocampus, and NTS [99].

1.3.3 Structure of ERs

ERα and ERβ are not true isoforms, but instead are two distinct proteins encoded by individual genes located on different chromosomes. In humans chromosome 6 encodes ERα, whereas chromosome 14 encodes ERβ [102]. ERα and ERβ protein structures share many similarities (Figure 1-1b). Like all members of the nuclear receptor superfamily they contain a DNA-binding domain, in which they share 97% sequence identity, and a ligand-binding domain, in which they share 55% sequence identity [94]. ERα and ERβ also contain an N-terminal ligand-independent transcriptional activation function domain (AF-1) that interacts with other transcription factors and a C-terminal AF-2 ligand-dependent transcriptional activation function domain that binds to coactivator proteins [102-104]. The AF-1 domain of ERα is very active while that of ERβ has minimal

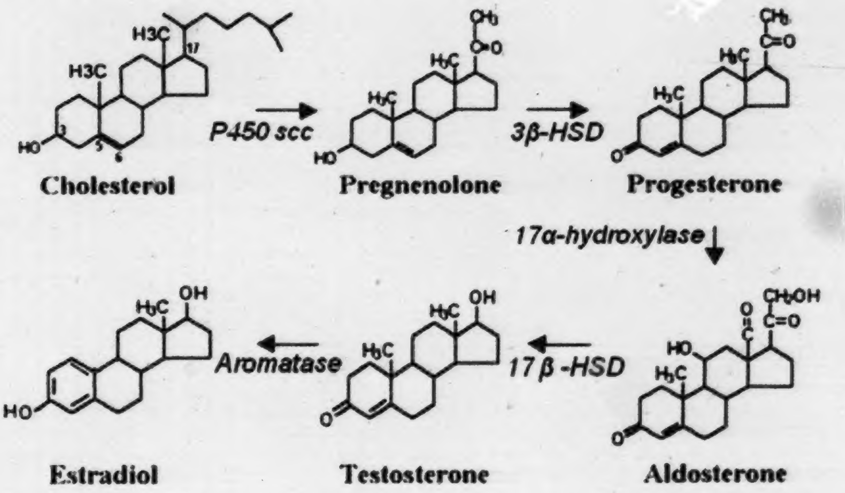
H3C
H3C
HO
Cholesterol
P450 scc
Pregnenolone
3β-HSD
Progesterone
17α-hydroxylase
Aldosterone
17β-HSD
Testosterone
Aromatase
Estradiol

Figure 1-1a. Biosynthetic pathway of estradiol synthesis. 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase. Modified from [105]

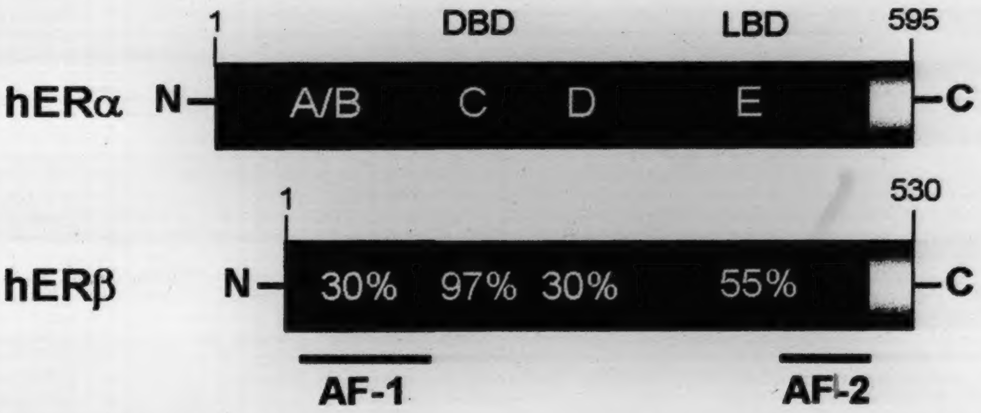
1
DBD
LBD
595
hERα
N
A/B
C
D
E
C
1
530
hERβ
N
30%
97%
30%
55%
C
AF-1
AF-2

Figure 1-1b. Structural domains of the human ERα and ERβ proteins. Domain A/B located at the N-terminus contains the activation function domain-1 (AF-1). Domain C is the DNA-binding domain (DBD). Domain E is the ligand-binding domain (LBD) and contains AF-2. Domain F is located at the C-terminus. The percentage of amino acid homology between ERα and ERβ in each domain are shown.

activity [102-104]. Splice variants of ERα and ERβ have been identified, but their biological significance is not well understood [102, 104, 106]. As a member of the G protein-coupled receptor family, GPR30 contains seven hydrophobic domains anchoring it into the plasma membrane and is bound to the heterotrimeric G-protein complex [107]

.

1.4 Estrogen signalling

ERs have been classically known for their role as transcription factors that act in the nucleus to regulate "genomic" actions of estrogen on target genes. However, a myriad of data over several years has focused on the role of ERs as "non-genomic" signalling molecules that carry out rapid actions of estrogen.

1.4.1 Genomic signalling

In the absence of a ligand, inactive nuclear ERs exist in an inhibitory complex with heat shock proteins [108-110]. Upon binding of estrogen to the ligand binding domain of the ER, a conformational change in the ligand binding domain occurs through a series of events that includes the dissociation of the ER from heat shock proteins and phosphorylation of the ER [103, 109, 110]. The conformational change induces either homodimerization or heterodimerization of ERs and allows the DNA binding domain of the ERs to bind to specific estrogen response elements (EREs) located in the promoter region of target genes [103, 108-111]. Once the ER-ERE complex is formed, coactivator proteins of the p160 family are recruited and help to stabilize the interaction between the ER-ERE complex [108, 111]. Coactivator proteins then bind to and activate the activation function domains of the ER, AF-1 and AF-2, inducing transcriptional activation or repression of target genes [102, 103] (Figure 1-2a).

Approximately 35% of human genes known to be regulated by estrogen do not contain ERE-like sequences [112]. Instead, these genes are regulated by estrogen through an ERE-independent mechanism where the ER does not directly bind to the target DNA, but, alternatively binds to and modulates the function of other transcription factors [111, 112] (Figure 1-2b). For example, upon E2 binding to the ER, the ER dimer interacts with the activator protein-1 (AP-1) transcription factor complex which consists of the transcription factors Fos and Jun. E2 induces the ER/AP-1 complex to regulate transcription of genes containing AP-1 sites, such as insulin-like growth factor-1 [111, 112]. ERs have been shown to bind to the AP-1 complex by two distinct mechanisms. Upon E2 treatment ER dimers bind to the AP-1 complex through the AF-1 domain, leading to enhanced transcriptional activity of target genes [113, 114]. On the other hand, selective estrogen receptor modulators, such as tamoxifen, induce ER dimers to bind to the AP-1 complex independently of the AF domains, leading to modulation of transcriptional activity of target genes [113, 114]. In addition, ERα and ERβ differentially regulate AP-1 transcription following E2 treatment, as ERα induces transcription while ERβ represses transcription of AP-1 sites [115]. In addition, genes that have GC-rich promoter sequences, such as c-FOS, are regulated by E2 through the interaction of the ER dimer with the Sp-1 transcription factor [111, 116, 117].

1.4.2 Non-genomic signalling

Estrogen elicits a variety of rapid effects that cannot be explained by genomic signalling where changes in gene transcription and protein synthesis occur. Instead, E2 initiates rapid effects by binding to membrane or cytoplasmic ERs leading to the activation of downstream signal transduction pathways [111, 118] (Figure 1-3). Some of

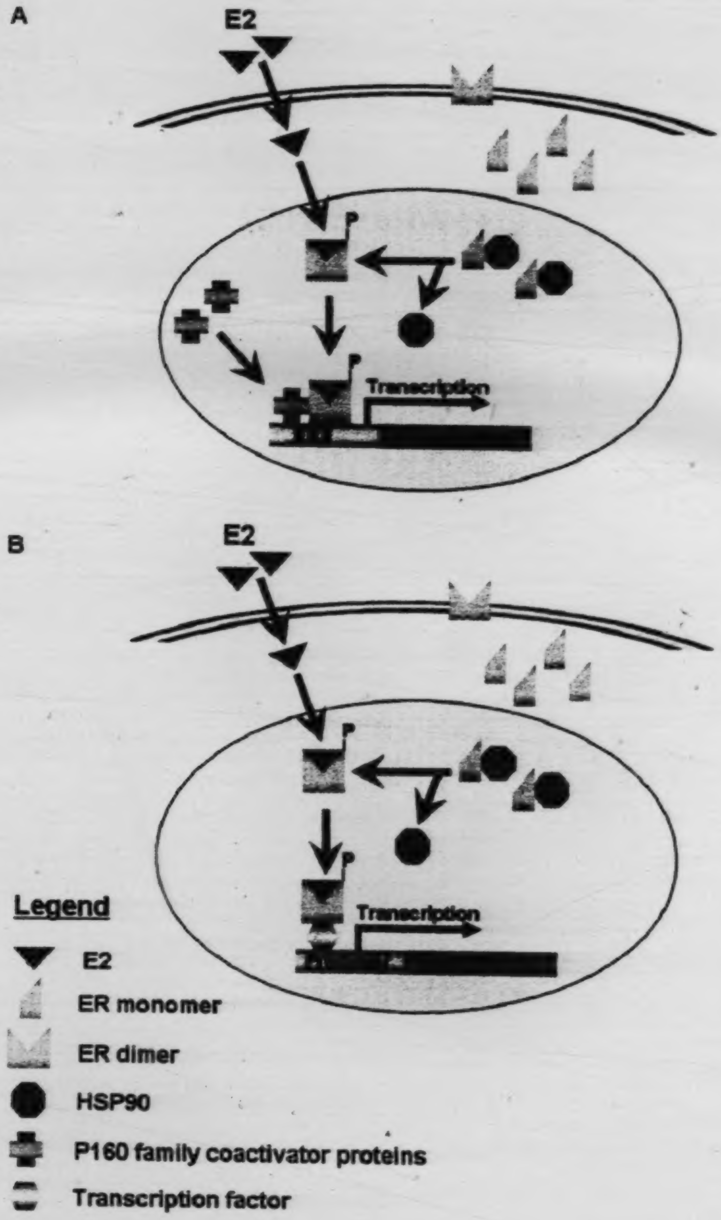
A
E2
P
Transcription
B
E2
P
Transcription
Legend
E2
ER monomer
ER dimer
HSP90
P160 family coactivator proteins
Transcription factor

Figure 1-2. Schematic representation of (A) ERE-dependent genomic signalling and (B) ERE-independent genomic signalling, leading to up- or down-regulation of gene transcription.

the rapid effects of E2 include the increase in intracellular Ca^{++} [58, 119], cyclic adenosine monophosphate (cAMP) [120] and nitric oxide (NO) production [121, 122], and the activation of the steroid receptor co-activator (Src) [58, 123], the mitogen-activated protein kinase (MAPK) [55, 119], and the phosphatidylinositol-3 kinase (PI3K) pathways [122, 124]. Rapid non-genomic actions of E2 are mediated by membrane ERs [111, 118]. Classical ERs are present at the plasma membrane of breast cancer cells [125], neurons [126], and in invaginations of the plasma membrane known as caveolae, in endothelial cells [125, 127]. Because ERs lack a trans-membrane domain, the mechanism through which ERs localize to the plasma membrane is not well understood. Recent studies have demonstrated that palmitoylation of ERα [128] and ERβ [129] induces their localization to the plasma membrane in human cancer cells (HeLa) and in Chinese hamster ovary cells, respectively. Furthermore, studies in human cancer cells have shown that that palmitoylation of ERα [128] and ERβ [130] induce their association with the plasma membrane protein, caveolin-1. Although non-genomic E2 signalling has received a great deal of attention in the past five years, the specific molecular mechanisms involved in mediating the rapid effects of E2 are largely unknown.

GPR30 has also been shown to mediate rapid effects of E2 including the mobilization of calcium [99, 107, 131] and an increase in cAMP activity [97, 107, 132]. The subcellular localization of GPR30 is a controversial topic, as some report it to be localized to the plasma membrane [107, 133] while others report that GPR30 is found in the endoplasmic reticulum membrane [131]. Recently it has been proposed that newly synthesized GPR30 is localized in the endoplasmic reticulum before being shuttled to the plasma membrane to carry out its functions [98, 134].

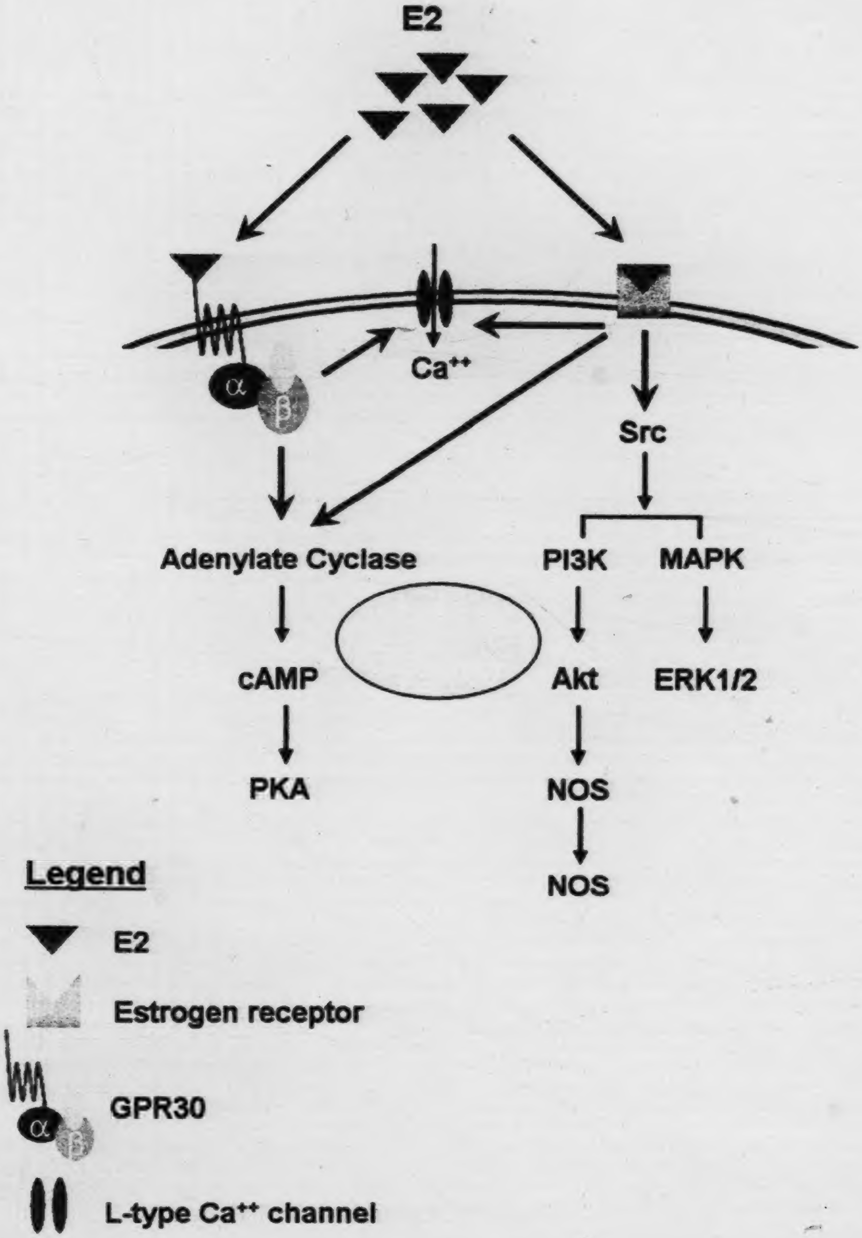
E2
Ca⁺⁺
Src
Adenylate Cyclase
PI3K
MAPK
cAMP
Akt
ERK1/2
PKA
NOS
NOS
Legend
E2
Estrogen receptor
GPR30
α
β
L-type Ca⁺⁺ channel

Figure 1-3. Schematic representation of non-genomic signalling initiated by E2 binding to membrane ERs and the downstream signal transduction pathways activated by E2.

1.4.3 Convergence of genomic and non-genomic signalling

While the two mechanisms for estrogen signalling have been studied and presented here separately, the prospect of these two pathways converging would allow for estrogen to meticulously control its cellular effects [111]. Several points of conversion have been proposed.

Because the phosphorylation of transcription factors by protein kinases often regulates their activity, E2 through non-genomic modulation of protein kinases may indirectly regulate the activity of transcription factors [111, 135]. The regulation of transcription factor activity would allow for estrogen to modulate genes that are not direct targets of estrogen action. For example, E2 rapidly activates the MAPK pathway which leads to downstream activation of the transcription factor, cAMP response element binding protein (CREB), which leads to expression of genes containing cAMP response elements (CREs) [111, 136]. In addition, E2 activates MAPK leading to AP-1- induced regulation of target genes [111, 113]. Furthermore, because the transcription factor, nuclear factor-κB (NF-kB), is phosphorylated by PI3K, non-genomic activation of PI3K by E2 indirectly increases the expression of target genes containing NF-κB binding sites [111, 137].

Another point of convergence includes the modulation of ER function through the non-genomic actions of E2. Because E2 activates the MAPK pathway, which along with other kinase pathways, phosphorylates ERs and increases their activity, E2 indirectly modulates the activity of ERs [111, 138, 139].

1.5 Estrogen signalling in neurons

The signal transduction pathways initiated by E2 in neurons have received a great deal of attention recently. However, compared to the depth of knowledge about E2 signal transduction in endothelial cells, our understanding of E2 signalling in neurons is in its infancy. To date, most investigations on E2 signalling in neurons simply implicate protein kinase pathways as mediators of E2's effects. However, the identities of specific signalling molecules and events involved in carrying out E2's effects are largely unknown. E2 has been shown to activate the MAPK pathway in cortical explants [140], and the PI3K pathway in midbrain neurons [141] and NG-108 neuroblastoma cells [142]. A recent study by Mannella and Brinton demonstrated that E2 increases the activity of MAPK and PI3K in the same population of cortical neurons [143].

Studies have shown that activation of MAPK mediates the neuroprotective effects of E2 against ischemia in CA1 hippocampal neurons [144] and β amyloid toxicity in the hippocampal cell line, HT22 [145]. In cortical neurons, E2 provides neuroprotection against hydrogen peroxide-induced toxicity by attenuating the hyperactivation of MAPK [146]. On the other hand, studies have demonstrated that activation of the PI3K pathway mediates the neuroprotective effects of E2 in the hippocampus against ischemia [147] and in cortical neurons against glutamate excitotoxicity [54].

1.6 Nitric oxide (NO)

It is firmly established that NO is a key mediator of the effects of E2 in endothelial cells. NO, through its potent vasodilator and anti-inflammatory actions, critically modulates vascular function [148, 149]. While NO is known to play an important role in the brain [150, 151], its role as a mediator of E2's effects in neurons is still poorly understood.

1.6.1 NO production

NO is a highly diffusible gaseous free radical that, amongst other physiological functions, acts as a neurotransmitter in the central nervous system [150, 152]. NO is produced when the enzyme nitric oxide synthase (NOS) and the co-substrates, nicotinamide adenine dinucleotide phosphate (NADPH) and O_2, oxidize the amino acid L-arginine to produce L-citrulline and NO [150, 151] (Figure 1-4). Three isoforms of NOS are expressed in a variety of cell types, neuronal NOS (nNOS) found primarily in neurons but also in skeletal and cardiac myocytes, endothelial NOS (eNOS) found primarily in endothelial cells but also in cardiomyocytes and platelets, and inducible NOS (iNOS) found primarily in macrophages and microglia [151, 153]. In addition, it was recently observed that eNOS [154, 155] and nNOS [155] are expressed in astrocytes. nNOS and eNOS are constitutively active and require Ca^{++}/calmodulin for their activation [151, 152]. NO production is tightly regulated through posttranslational modification of NOS, which includes phosphorylation and protein-protein interactions [156, 157]. Phosphorylation sites have been identified on eNOS and nNOS. An increase in the levels of phosphorylation at site Ser^{1177} on eNOS or Ser^{1412} on nNOS leads to an increase in enzyme activity, whereas an increase in phosphorylation levels at Thr^{495} on eNOS or Ser^{847} on nNOS lead to a decrease in enzymatic activity [158, 159]. Heat-shock protein 90 (HSP-90) has been shown to bind to and increase the activity of both eNOS [156, 157] and nNOS [156]. Once produced, NO diffuses into adjacent cells to act on second messenger pathways. The best characterized effect of NO production is activation

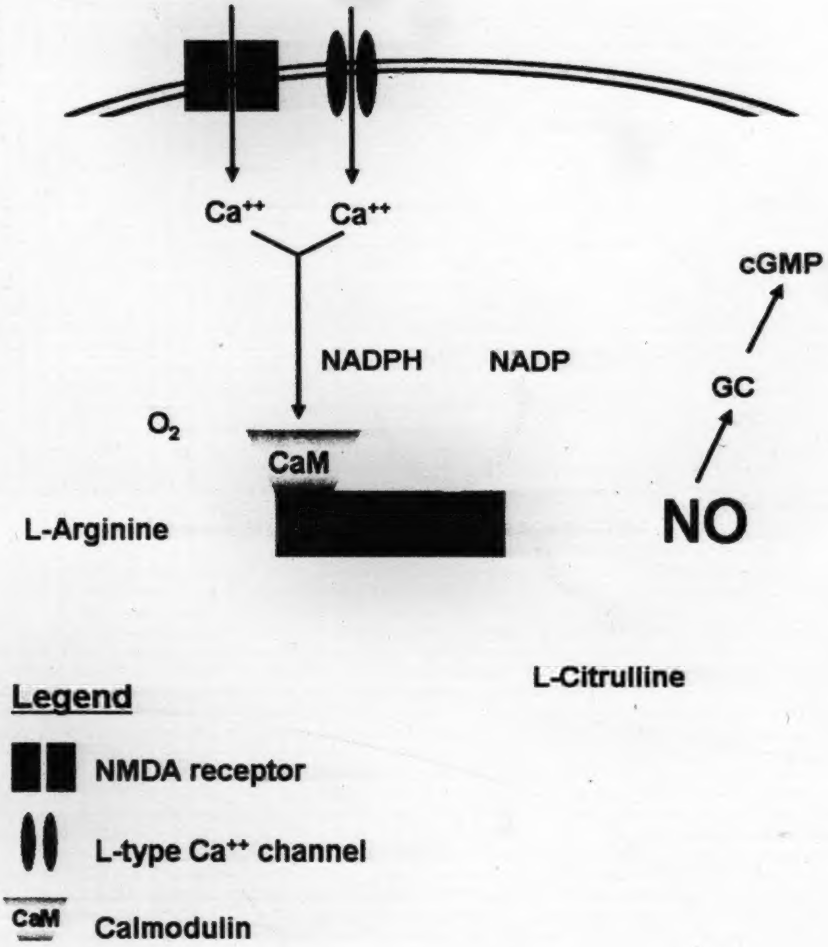
Ca++
Ca++
cGMP
NADPH
NADP
GC
O2
CaM
L-Arginine
NO
L-Citrulline
Legend
NMDA receptor
L-type Ca++ channel
CaM
Calmodulin

Figure 1-4. Schematic representation of NO production. NO is produced by the oxidation reaction of L-arginine to L-citrulline by NOS and the co-substrates, nicotinamide adenine dinucleotide phosphate (NADPH) and O_2.

of soluble guanylyl cyclase which leads to the production of cyclic guanosine monophosphate (cGMP) [150, 160].

1.6.2 Actions of NO in the CNS

NO is an important neurotransmitter in the CNS that is involved in modulating neuronal function. NO depolarizes neurons of the PVN through a cGMP-dependent mechanism [161]. NO also increases the firing rate of neurons in the dorsal motor nucleus of the vagus in brain stem slices [162]. NO facilitates the induction of long-term potentiation (LTP), as inhibitors of NO block the induction of LTP in hippocampal slices [163] and in CA1 hippocampal neurons *in vivo*. Not only does NO enhance LTP, but it has been suggested that NO production is necessary for LTP in hippocampal neurons [164]. NO has also been shown to modulate the release of a variety of neurotransmitters. In the cerebral cortex of guinea pigs NOS inhibitors blocked the N-methyl-D-aspartic acid (NMDA)-induced release of norepinephrine and L-glutamate [165]. Also, the addition of exogenous NO induces the release of dopamine in primary ventral mesencephalic neuronal cultures [166] and the release of acetylcholine in the basal forebrain of conscious rats [167].

An important and well studied role of nitric oxide is its effects in the autonomic nervous system [150, 152]. NO-producing neurons are expressed in many autonomic nuclei including the paraventricular nucleus, the nucleus of the solitary tract, and the ventrolateral medulla [168]. Autonomic neurons of the PVN that project to the rostral ventrolateral medulla (RVLM) pressor region, express nNOS [169]. NO is an important regulator of sympathetic activity. Chronic administration of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME), in the drinking water of rats leads to an increase in resting blood pressure and heart rate [170]. Intravenous injections of NO donors lead to acute decreases in blood pressure [171], while the NO inhibitor, L-NAME, increases blood pressure [172]. Endogenous NO in the CNS has been suggested to have a tonic effect on sympathetic output. Intracerebroventricular (I.C.V.) injections of NOS inhibitors lead to an increase in blood pressure and heart rate in anesthetized rats [173]. I.C.V. injection of the NO precursor, L-arginine, induces a decrease in blood pressure and abdominal sympathetic nerve activity [174]. When injected directly into the PVN the NO donor, sodium nitroprusside, decreases blood pressure, heart rate, and renal sympathetic nerve activity, while injection of the NOS inhibitor L-NAME increases these parameters [175].

In the brain NO also plays an important role in modulating the stress response. Restraint stress increases the activity of NO-producing neurons in many regions of the brain including the PVN of male rats [176]. Levels of the NO by-product, nitrite, are increased in the PVN following immobilization stress in male rats [177]. NO in the brain has also been shown to mediate the effects of E2 on blood pressure responses to restraint stress [86]. I.C.V. injection of a NOS inhibitor blocks the attenuated blood pressure response to restraint stress in E2-treated OVX (OVX-E) rats [86]. In addition, restraint stress increases the levels NO in the hypothalamus and brainstem of OVX-E rats, but not in vehicle-treated OVX rats [86].

1.6.3 Effects of estrogen on NO in endothelial cells

Most of our current understanding about the role of NO as a mediator of E2's effects comes from experiments performed in endothelial cells. Prolonged E2 treatment has been shown to increase protein expression levels of eNOS in cerebral microvessels

[178], human endothelial cells [179], and in pial arteries of OVX rats [180]. Prolonged E2 treatment also increased eNOS mRNA levels in ovine endothelial cells [181]. The promoter region of eNOS contains EREs, an SP-1 motif, and AP-1 binding sites [158, 182], suggesting that E2 can increase eNOS transcription and subsequent protein levels through multiple genomic mechanisms.

E2 also induces rapid non-genomic effects on eNOS. E2 has been shown to rapidly increase eNOS phosphorylation [122, 183], eNOS activity [184], and NO production [122, 183]. Using membrane impermeable E2-conjugated bovine serum albumin (BSA), E2 has been shown to activate membrane ERs, leading to an increase eNOS phosphorylation [183, 185] and eNOS activity in endothelial cells [183, 186]. Activation of the MAPK and PI3K pathways has been shown to mediate the effects of E2 on eNOS activity in endothelial cells. MAPK was found to mediate the E2-induced increases in eNOS phosphorylation [183], eNOS activity [183, 187] and NO production [183]. PI3K has been shown by many groups to mediate E2-induced increases in eNOS phosphorylation [122, 124], eNOS activity [124, 188], and NO production [122, 124, 185] in endothelial cells.

Endothelial cells contain ERα and ERβ [127, 187, 189]. However, most of the literature regarding the effects of E2 on NO in endothelial cells focuses on the role of ERα [148, 188, 190]. It was recently reported that in female rat cerebral microvessels, the gene and protein expression levels of ERβ are approximately 3-fold higher than ERα [191] and that ERβ gene expression is 10-fold higher than ERα and GPR30 in human arteries and veins [192]. Fortunately, the role of ERβ in the effects of E2 on NO in endothelial cells can now be distinguished, as selective ligands for ERβ have been recently developed. Using these selective ERβ ligands, two groups have demonstrated that E2 acts in part through ERβ to increase eNOS activity and NO production in endothelial cells [127, 187]. GPR30 gene expression was also recently identified in human blood vessels [192]. However, the role of GPR30 in the effects of estrogen on NO in endothelial cells has yet to be determined.

1.6.4 Effects of estrogen on NO in neurons

Although NO is a well known mediator of E2's effects in endothelial cells, it is becoming increasingly evident that NO is also a key mediator of the effects of E2 in neurons. Chronic E2 treatment leads to increased levels of nNOS expression in the preoptic nucleus [193], ventromedial hypothalamus [194], and the PVN [195] of OVX rats. It is important to note that OVX rats express lower levels of nNOS expression in the PVN compared to intact females and E2 treatment increases nNOS expression to levels equal to intact female rats [195]. Chronic E2 treatment also increases nNOS mRNA levels in the ventral medial hypothalamus [196] and in the hippocampus of OVX rats [197]. NO production is higher in preoptic [198] and median eminence explants [199] from cycling female rats during proestrus, when circulating levels of E2 are high, than during diestrus, when levels of E2 are low. Acute E2 treatment has been shown to increase NOS activity [121]. E2 also rapidly increases NO production from neuroblastoma cells [121, 200] and from median eminence explants of rats [201].

In addition to the effects of E2 on nNOS in the brain, E2 has also been shown to modulate eNOS expression in the cerebral vasculature. Specifically, E2 treatment increases eNOS protein expression in cerebral microvessels [178, 202], pial arteries [180, 203], and *ex vivo* preparations of the median eminence [204] of OVX rats.

NO in the brain has been shown to mediate the attenuation of blood pressure responses to restraint stress by E2 in OVX rats [86]. NO also mediates the neuroprotective effects of E2 against hydrogen peroxide toxicity [200] and serum deprivation in neuroblastoma cells [205]. Furthermore, NO mediates the E2-induced increase in hippocampal dendritic branching [206].

1.7 Focus of thesis

NO is a key mediator of E2's effects in the cardiovascular system and it is becoming increasingly clear that NO mediates many effects of E2 in the CNS. Although it has been demonstrated that E2 acts in the brain through NO to attenuate blood pressure responses to restraint stress [86], the specific brain region in which E2 acts is unknown. A likely candidate is the PVN, as it is an autonomic nucleus that regulates sympathetic output and expresses ERβ. Therefore, the first objective of this thesis was to investigate the effects of E2 on the NO system in the PVN. Specifically, the hypothesis that E2 acts in the PVN to alter NOS expression through an ERβ-dependent mechanism was tested (Chapter 3). Because these experiments demonstrate that E2 acts in the PVN to modulate the NO system, my next objective was to determine the effects of E2 in the PVN on blood pressure and heart rate and to explore the potential role of NO in these effects. Therefore, the hypothesis that E2 injected into the PVN attenuates blood pressure responses to L-glutamate stimulation through ERβ and NO was tested (Chapter 4). While it is clear that E2 acts on NO in neurons, the signalling pathways leading to activation of the NO system are not well understood. Because my experiments (Chapter 3 and 4) suggest that E2 through ERβ acts on NO in the PVN, the final objective was to characterize the signal transduction pathway through which activation of ERβ in neurons affects the NO system. In particular, I tested the hypothesis that activation of ERβ increases NO production in neurons by increasing nNOS activity through changes in nNOS phosphorylation (Chapter 5).

Chapter 2

Materials and Methods

2.1 List of chemicals and reagents

Name of Reagent	Source of Reagent
17β-estradiol (E2)	Sigma
2,3-diaminonaphthaline (DAN)	Molecular Probes
3,3-diaminobenzidine tetrahydrochloride	Sigma
4'-6-diamidino-2-phenylindole (DAPI)	Vector
7-nitroindazole monosodium salt (7-NiNa)	A.G. Scientific
^{35}S-UTP	Perkin Elmer/NEN
Aprotinin	Sigma
*Bam*HI	Promega
Basal medium eagle (BME)	Invitrogen
Bicuculline methiodide (BIC)	Sigma
Ciliary neurotrophic factor (CNTF)	Cedarlane
Cresyl violet	Sigma
CTP/GTP/ATP	Invitrogen
Cytosine arabinoside	Sigma
Cytoseal	Richard-Allen Scientific
D-glucose	Invitrogen
Diarylpropionitrile (DPN)	Tocris
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol DTT	Invitrogen
dNTP	Invitrogen
Evans blue	Sigma-Aldrich
Fetal bovine serum (FBS)	Invitrogen
FK506	Sigma-Aldrich
Genistein	Sigma
Glutamax	Invitrogen
Halothane	Halocarbon
Hank's balanced salt solution	Invitrogen
Heat inactivated horse serum	Invitrogen
HEPES buffer solution	Invitrogen
ICI 182,780	Tocris
L-glutamate	Sigma
L-glutamic acid	Sigma
Lipopolysaccharide (LPS)	Sigma
Mercaptoethanol	Sigma-Aldrich
Methyl-piperidino-pyrazole (MPP)	Obiter Research/Tocris
N2 supplement	Invitrogen
N5-(1-Iminoethyl)-L-ornithine (L-NIO)	Tocris Cookson
N-nitro-L-arginine methyl ester (L-NAME)	Sigma-Aldrich
Neomycin	Invitrogen
Neurobasal medium	Invitrogen
Neurobasal-A medium	Invitrogen
Neutral red	Allied Chemical

Nickel ammonium sulphate	Sigma
Nonfat dry milk	Carnation
NTB photographic emulsion	Kodak
Paraformaldehyde	Sigma-Aldrich
Penicillin-streptomycin	Invitrogen
Pepstatin A	Sigma
Phenylmethylsulfonyl fluoride	Sigma
Poly-D-lysine	Sigma
Propyl-pyrazole-triol (PPT)	Tocris
Protein phosphatases inhibitor cocktail 1	Sigma
R,R-tetrahydrochrysene	Tocris
Rabbit serum	Vector Labs
RNase inhibitor	Invitrogen
Sodium bicarbonate	Invitrogen
Sodium deoxycholate	Sigma
Sodium dodecyl sulfate	Si
Sodium orthovanadate	Sigma
Sodium pyruvate solution	Invitrogen
T7 polymerase	Promega
Tergitol	Sigma
Triton-X 100	Sigma
Trypsin	Invitrogen
Tween-20	Sigma
Urethane	Sigma
Vectastain Elite ABC detection kit	Vector Labs
Western Lightning chemiluminescence kit	PerkinElmer

2.2 List of primary antibodies

Specificity	Dilution	Source
Rabbit anti-eNOS	1:1000	Santa Cruz
Rabbit anti-nNOS	1:8000	Chemicon
Rabbit anti-iNOS	1:2500	Biolmol Research Labs
Mouse anti-RECA-1	1:100	Serotec
Chicken anti-ERβ	1:1000	Provided by Dr. Gustafsson, Sweden
Mouse anti-MAP-2	1:100	Gene Tex
Rabbit anti-GFAP	1:100	Abcam
Rabbit anti-phospho nNOS (Ser^{1412})	1:500	Abcam
Rabbit anti-nNOS	1:500	Santa Cruz
Rabbit anti-phospho Src (Tyr^{416})	1:1000	Cell Signalling

Rabbit anti-Src	1:3000	Santa Cruz
Rabbit anti-phospho Akt (Ser^{473})	1:1000	Cell Signalling
Rabbit anti-Akt	1:1000	Cell Signalling
Rabbit anti-phospho ERK1/2	1:2000	Cell Signalling
Rabbit anti-ERK1	1:2000	Santa Cruz
Mouse anti-β-actin	1:4000	Jackson Immunoresearch

2.3 List of secondary antibodies

Specificity	Dilution	Source
Biotinylated goat anti-rabbit	1:300	Vector Laboratories
Biotinylated rabbit anti-chicken	1:200	Jackson Immunoresearch
Cy3-donkey anti-rabbit IgG	1:2000	Jackson Immunoresearch
FITC-donkey anti-mouse IgG	1:2000	Jackson Immunoresearch
Donkey anti- rabbit Alexa Fluor 488	1:500	Invitrogen
Donkey anti- mouse Alexa Fluor 647	1:500	Invitrogen
Chicken anti-rhodamine	1:2500	Jackson Immunoresearch
Goat anti-mouse HRP	1:2000	Jackson Immunoresearch
Donkey anti-rabbit HRP	1:2000	Jackson Immunoresearch

2.4 Animals

Male and female Sprague-Dawley rat pups 5-7 day old, used to obtain hypothalamic slice cultures, were purchased from the Biological Sciences Animal Center and the University of Alberta. Male Sprague-Dawley rats (250-350g), used for microinjection studies were purchased from the Biological Sciences Animal Center at the University of Alberta. Pregnant female Sprague-Dawley rats (day 17 of gestation), used to obtain primary cultures, were purchased from Charles River Laboratories (Saint-Constant, Quebec). Male and pregnant female rats were housed at 21°C in a 12:12 hour light-dark cycle and were fed *ad libitum*. All experimental procedures were approved by Health Sciences Lab Animal Services at the University of Alberta.

2.5 Pharmacological Agents

The pharmacological agents used in the studies described in this thesis include 17β-estradiol (E2, Sigma-Aldrich); the non-selective ER antagonist, ICI 182,780 (Tocris); the ERβ agonists, genistein (Sigma-Aldrich) and diarylpropionitrile (DPN, Tocris), which are 20-fold [207, 208] and 70-fold [209] more selective for ERβ than ERα respectively; the ERα agnonist, propyl-pyrazole-triol (PPT, Tocris), which is 1000- fold more selective for ERα than ERβ [209]; the pure ERβ antagonist R,R-tetrahydrochrysene (R,R-THC, Tocris) [209]; and the ERα antagonist, methyl-piperidino-pyrazole (MPP Tocris), which is 200-fold more selective for ERα than ERβ [209]. Concentrations of these agents were chosen based on the relative effective potencies of each compound in relation to the concentration of E2 used, as demonstrated by dose–response experiments performed in human endometrial cancer (HEC-1) cells [209-211].

Other pharmacological agents used include the non-selective NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich) with an IC_{50} of 500 nM; the NOS inhibitor, N5-(1-Iminoethyl)-L-ornithine (L-NIO, Tocris), with an IC_{50} of 500 nM and which is five to ten times more potent as an inhibitor of eNOS than nNOS or iNOS [172]; the selective nNOS inhibitor 7-nitroindazole sodium salt (7-NiNa, A.G.Scientific), with an IC_{50} of 47 nM; and the $GABA_A$ receptor antagonist, bicuculline (Sigma). Effective concentrations of L-NAME, 7-NiNA, L-NIO, and bicuculline were chosen based on a previous microinjection study from this laboratory [212]. Finally, the Src inhibitor, PP2 (Calbiochem, San Diego); the inactive analog of PP2, PP3 (Calbiochem); the PI3K inhibitor, LY294002 (Cayman Chemical, Ann Arbor); and the MAPK inhibitor, PD98059 (Cayman Chemical) were used in a study described in this thesis.

2.6 Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an ERβ-dependent mechanism in hypothalamic slice cultures

2.6.1 Hypothalamic slice culture

Hypothalamic slice cultures were prepared as described by others [213]. Briefly, 5-7 day old Sprague Dawley rat pups were quickly decapitated. Blocks of hypothalamic tissue were cut from the brains and sectioned at a thickness of 400 μm using a McIlwain tissue chopper (Mickle Laboratory Company, UK). Slices were immediately placed into Gey's balanced salt solution (4°C) enriched with 5 mg/L glucose. A total of 5 coronal slices from each brain was placed on a single Millicell-CM filter insert (Millipore, pore size 0.4 μm, diameter 30 mm). Each filter insert was placed into a 35 mm petri dish containing 1.1 mL of serum-containing medium (SCM); each dish, representing an *N* of 1, was exposed to a single treatment as described below.

Slice cultures were maintained in a humidified incubator at 35°C in 5% CO_2 and 95% air. Hypothalamic slice cultures were maintained in SCM for 7 days. SCM was composed of 50% basal medium eagle (BME, Invitrogen), 25% heat-inactivated horse serum (Invitrogen), 25% Hank's balanced salt solution (HBSS, Invitrogen), 1 mM L-glutamine (Invitrogen), 5 mg/L glucose, 25 μg/mL penicillin-streptomycin, 50 μg/mL neomycin (Invitrogen) and 25 μg/mL ciliary neurotrophic factor (CNTF, Cedarlane). Culture medium was then changed to serum-free medium (SFM) on day 8. SFM was composed of 95% phenol red-free Neurobasal-A medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM Glutamax (Invitrogen), 10 mM HEPES (Invitrogen), 0.075% sodium bicarbonate (Invitrogen), 5 mg/mL glucose, 25 μg/mL penicillin-streptomycin and 50 μg/mL neomycin. All media were changed 3 times a week. Slice cultures were subjected to different experimental conditions on day 9.

2.6.2 Expression of eNOS and nNOS in hypothalamic slice cultures

To determine which NOS isoforms are present in hypothalamic slice cultures, immunohistochemistry for eNOS, nNOS and inducible NOS (iNOS) was performed after 8 days in culture. To verify that eNOS expression was localized in blood vessels, slice cultures were double-labelled with eNOS and rat endothelial RECA-1, a marker for rat endothelial cells [214]. Hypothalamic slice cultures were not expected to basally express iNOS, as it is typically expressed in the brain only after an immune challenge. For this reason a positive control for iNOS expression was performed in which slice cultures were treated with lipopolysaccharide (LPS) (1 μg/mL) for 24 hours.

Hypothalamic slice cultures were fixed for 1 hour in ice-cold 4% paraformaldehyde (PFA, in PBS) and then incubated for 48 hours at 4°C in primary antibody (in 0.4% Triton/PBS). The following primary antibodies were used: rabbit anti-eNOS (1:1000), rabbit anti-nNOS (1:8000), and rabbit anti-iNOS (1:2500). Slice cultures were incubated in biotinylated goat anti-rabbit antibody (1:300, in 0.4% Triton/PBS) for 1 hour at room temperature. Slice cultures were then incubated in ABC solution (Vectastain Elite ABC kit, Vector labs) for 1 hour at room temperature. The reaction product was visualized with a 5-10 min incubation in the chromagen, 3,3'diaminobenzidine tetrahydrochloride (DAB, Sigma, 5 mg/10 mL, in PBS).

Slice cultures labelled by immunofluorescence were incubated for 48 hours at 4°C in the following primary antibodies: rabbit anti-eNOS (1:1000) and mouse anti-RECA-1 (1:100). Slice cultures were incubated in the following secondary antibodies: CY3-conjugated donkey anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG (1:2000).

Individual slices were gently removed from filters with a fine-tipped paintbrush and mounted onto slides. Slides were then rinsed in water, allowed to dry thoroughly and cover slipped using Cytoseal.

2.6.3 Quantification of NOS expression levels

NOS expression in the PVN was quantified from one hypothalamic slice per culture dish (which translates to one slice per animal). Photographs were acquired with Image-Pro Plus software (Media Cybernetics, Silver Springs, MD) using a DC-330 camera (DAGE-MTI, Michigan City, IN) mounted onto a Leica microscope.

Quantification of eNOS expression was performed using Scion Image software (NIH). Images were converted into grey scale and thresholded to remove background; the density of eNOS-immunoreactive (eNOS-IR) elements in the PVN was determined by counting the numbers of pixels above threshold in a defined area, as previously described by others [215]. When the PVN was analyzed as a whole, eNOS expression was quantified within a standardized box which encompassed one side of the PVN and values for both sides were summed to obtain a value for the entire PVN; when the PVN was divided into regions (see below), a separate standardized box was used for each region. nNOS-IR neurons were counted either within the entire PVN or within regions (see below).

The PVN of the hypothalamus is an autonomic center comprised of two functionally distinct regions, the parvocellular region which regulates the autonomic and neuroendocrine systems [152, 216] and the magnocellular region which regulates posterior pituitary function [208, 216]. In order to determine if E2 affects the NO system differentially within the parvocellular and/or magnocellular regions, NOS expression in these regions was analyzed separately in some experiments. The parvocellular and magnocellular regions were delineated based on anatomical location; eNOS expression and numbers of nNOS-IR neurons within each region of the PVN were interpreted as proportions of the total signal within the PVN. To determine if E2 affects eNOS expression exclusively in the PVN within the slice, eNOS expression was quantified in an area adjacent to the PVN delineated by a 400 μm^2 box placed 100 μm away from the ventrolateral edge of the parvocellular region of the PVN.

2.6.4 Estrogen receptor expression

To study the expression of ERα and ERβ in the PVN of hypothalamic slice cultures, *in situ* hybridization and immunohistochemistry for ERα and ERβ, respectively, were performed after 8 days in culture. *In situ* hybridization was performed as follows. Hypothalamic slice cultures were fixed for 1 hour in ice-cold 4% PFA (in PBS), rinsed and mounted onto slides, and allowed to dry thoroughly. Slices were then immersed in ice-cold 4% PFA for 10 min, rinsed twice in PBS, incubated in proteinase K buffer for 8 min, rinsed in PBS and treated with 4% PFA for 4 min. Slices were incubated in acetic anhydride (in TE) for 10 min followed by 70% ethanol in sodium acetate, 80% ethanol in sodium acetate, 95% ethanol, 100% ethanol and allowed to air dry. Slices were hybridized overnight at 53°C in a humid chamber with a ^{35}S-labeled antisense RNA probe for ERα generated from a 365 bp cDNA fragment kindly provided by Dr. Martha Campbell-Thompson (University of Florida) and transcribed with T7 polymerase from a plasmid linerized with *Bam*HI. After hybridization, sections were rinsed twice in 2x SSC, followed by incubation in RNase A in STE buffer at 37°C for 30 min, 1x SSC at 45°C for 45 min, 0.1x SSC at 65 °C for 50 min, and then air dried. Slices were exposed to X-ray film (Kodak Biomax MR film, Kodak) for 3 days and then dipped in NTB-2 Kodak photographic emulsion (diluted 1:1 with water). Autoradiograms were exposed for 14 days and after processing, sections were stained with 0.5% cresyl violet (Sigma).

For ERβ immunohistochemistry, hypothalamic slice cultures were fixed for 1 hour in 3% acrolein (in 4% PFA), rinsed in PBS, incubated in 1% sodium borohydride for 20 min, rinsed in PBS, and incubated in 0.2% Triton-X PBS for 20 min and then in 0.1 M glycine for 30 min. Slices were then incubated in blocking solution (5% rabbit serum, 1% H_2O_2 in 0.2% Triton/PBS) for 30 min, and incubated in anti-ERβ primary antibody kindly provided by Dr. J.A. Gustafsson (Huddinge, Sweden) (503 IgY, 1:1000 in 0.2% Triton/PBS containing 5% rabbit serum) for 72 hours at 4°C. Slice cultures were incubated in biotinylated rabbit anti-chicken antibody (1:200, Jackson Immuno Research) for 1 hour at room temperature. Slice cultures were then incubated in ABC solution (Vectastain Elite ABC kit, Vector labs) for 1 hour at room temperature. The reaction product was visualized after a 5 –10 min incubation in the chromagen, DAB (Sigma, 5 mg/10 mL, in PBS) containing 5 mg of nickel ammonium sulphate. Individual slices were then mounted and coverslipped as described above.

2.6.5 Experimental Design

Dose response experiments were performed to observe the effects of 17β-estradiol (E2, Sigma), genistein (Sigma), and propyl-pyrazole-triol (PPT, Tocris) on eNOS and nNOS protein expression in the PVN. Hypothalamic slices were incubated in either E2 (0.1 nM to 100 nM, in SFM), genistein (1 nM to 1 μM, in SFM containing 0.1% DMSO), or PPT (1 nM – 1 μM, in SFM containing 0.1% DMSO) for 48 hours; controls received vehicle (SFM or SFM containing 0.1% DMSO).

Based on dose response data, slice cultures were incubated in 1 nM E2 or 0.1 μM genistein and were processed for eNOS, nNOS immunohistochemistry at 0, 1, 8, 24 and 48 hours. NOS expression was measured within the entire PVN at all time points, while NOS expression in the parvo- and magnocellular regions of the PVN was measured only at 24 hours as this time point was used in subsequent experiments. As PPT did not affect NOS expression, time course experiments using PPT were not performed.

To investigate whether E2 or genistein alters NOS expression through an ER-dependent or -independent mechanism, the non-selective ER antagonist, ICI 182,780 (Tocris, Ellisville, MO, USA), was used to block the effects of E2 or genistein on NOS expression. Slice cultures were incubated in ICI 182,780 (10 nM, in SFM) or ICI 182,780 plus E2 (1 nM) for 24 hours, while other slice cultures were incubated in ICI 182,780 (1 nM – 1 μM) or ICI 182,780 plus genistein (0.1 μM) for 24 hours.

To investigate the role of ERα in the effects of E2 and genistein on eNOS expression in the PVN, the selective ERα antagonist, methyl-piperidino-pyrazole (MPP) (Obiter Research, Urbana, IL, USA), was used. Slice cultures were incubated with MPP (10 nM –10 μM, in SFM), MPP plus E2 (1 nM), or MPP plus genistein (0.1 μM) for 24 hours. As ERα is present in blood vessels but not in neurons of the PVN [100, 191, 217], we chose to investigate the effect of MPP only on eNOS expression.

2.7 Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor β and NO

2.7.1 Microinjection surgeries

Male rats (250-300 g) were anesthetized with intraperitoneal injections of urethane (1.75 g/Kg, Sigma, St. Louis, MO) and body temperature was maintained at 37°C with a heating pad. Microinjection surgeries were carried out as previously described [212]. Briefly, the left femoral artery was cannulated with PE-50 tubing (Becton Dickson and Co. Sparks, MD), connected to a pressure transducer attached to the computational acquistition system, D1-150 RS (DATAQ instruments, Akron, OH). Rats were then placed in a stereotaxic frame where a guide cannula was then lowered into the PVN according to the co-ordinates, 6.8 mm anterior, 0.1 mm lateral, and 2.2 mm ventral to the interaural zero, as previously described [212]. Mean arterial pressure (MAP) and heart rate (HR) were recorded and allowed to stabilize for 20-30 minutes before starting each experiment at which time unilateral microinjections of solutions (100 nL) were made over 1 minute.

2.7.2 Experimental Protocol

Each animal was used in only one experiment and received a total of two microinjections into the PVN. The first injection varied with each experiment and is described below. MAP and HR were then measured for 30 minutes to determine the effects of each pharmacological agent alone on resting MAP and HR. Thirty minutes after the first injection, L-glutamate (50 nmol) was injected into the PVN. MAP and HR were measured for an additional 30 minutes to determine the effects of the pharmacological agent(s), delivered in the first injection, on the L-glutamate–induced increases in MAP and HR.

All pharmacological agents were freshly diluted in saline, except for MPP, which was diluted in water, from stock solutions prepared in ethanol or DMSO. The maximal amount of ethanol or DMSO present in solutions was 1%, except in the case of MPP, where 10% DMSO was present. Vehicle injections contained concentrations of ethanol or DMSO equal to those present in each drug-containing solution.

2.7.3 Effect of E2 microinjection into the PVN on L-glutamate-induced increases in MAP and HR

Animals received an injection of vehicle (saline or water; n=15) into the PVN and an injection of L-glutamate (50 nmol) 30 minutes later. Other animals received an injection of E2 (0.1, 1 or 10 pmol; n≥8 for each group) into the PVN followed by L-glutamate 30 minutes later.

2.7.4 Role of ERs in E2's effect on the L-glutamate-induced increase in MAP

Animals received an injection of one of the following: ICI 182,780 (10 pmol; n= 6), diarylpropionitrile (DPN; 5, 50 or 100 pmol; n≥6 for each group), PPT (10 or 100 pmol; n=7 for each group), MPP (1000 pmol; n=8) or R,R-tetrahydrochrysene (R,R-THC; 50 pmol; n=5) into the PVN followed by L-glutamate 30 minutes later. Other animals received an injection of E2 (10 pmol) in combination with one of the following: ICI 182,780 (10 pmol; n=7), MPP (1000 pmol; n=8) or R,R-THC (5 and 50 pmol; n≥ 5 for both groups) into the PVN followed by L-glutamate 30 minutes later.

2.7.5 Role of NO and GABA in E2's effect on the L-glutamate induced increase in MAP

Animals received an injection of one of the following: N-nitro-L-arginine methyl ester (L-NAME; 2000 pmol; n=7), 7-nitroindazole sodium salt (7-NiNa; 0.05 pmol; n=7) or N5-(1-Iminoethyl)-L-ornithine (L-NIO; 100 pmol; n=9) into the PVN followed by L-glutamate 30 minutes later. Other animals received an injection of E2 (10 pmol) in combination with one of the following: L-NAME (2000 pmol; n=6), 7-NiNa (0.05 pmol; n=6) or L-NIO (2000 pmol; n=8) into the PVN followed by L-glutamate 30 minutes later.

Other Animals received an injection of bicuculline (200 pmol; n=8) or E2 (10 pmol) in combination with bicuculline (200 pmol; n=7) into the PVN followed by L-glutamate 30 minutes later.

2.7.6 Data analysis

Baseline MAP and HR were calculated by averaging values for 5 minutes prior to the first injection. Peak changes in MAP or HR were determined by subtracting baseline values from the peak value reached within 30 minutes following injection. The area under the curve (AUC) was determined to measure amplitude over time by calculating the area between baseline and each amplitude value for 20 minutes following injection.

2.7.7 Verification of injection site

At the end of each experiment 1% Evan's Blue (Sigma) was injected into the PVN. Brains were removed and fixed in ice-cold 4% paraformaldehyde for 2 days. Brains were frozen and serial coronal sections (50 μm) were stained with neutral red (0.5%, Fisher Scientific, Pittsburgh, PA). The locations of the injection site were determined with light microscopy and only rats with injection sites located within the PVN were included in the data analysis.

2.8 Activation of ERβ increases NO production by increasing phosphorylation levels of nNOS at Ser^{1412} through a Src/PI3K/Akt-dependent pathway in hypothalamic neurons

2.8.1 Primary hypothalamic cultures

Primary hypothalamic neurons were cultured from embryonic day 17 rat pups using a modified version of our previously described protocol [218]. Briefly, hypothalami were dissected from brains posterior to the optic chiasm, anterior to the mammillary bodies and along the lateral sulcus to a depth of approximately 2 mm. Hypothalami were rinsed 3 times in Hank's balanced salt solution (HBSS, containing 15 mM Hepes and 30 μg/mL penicillin-streptomycin) followed by digestion with 0.125% trypsin for 15 minutes at 37°C. Hypothalamic tissue was rinsed with HBSS and trypsin was inactivated by adding fetal bovine serum (FBS) for 30 seconds. Tissue was rinsed again 3 times in HBSS and then mechanically dissociated by trituration in serum-containing medium (SCM) consisting of phenol-red free neurobasal medium composed of 10% FBS, 15 mM Hepes, 1.5 mM sodium pyruvate, 0.6 mM L-glutamine, and 30 μg/mL penicillin/streptomycin.

The differential adhesion method [219] was used to facilitate the removal of non-neuronal cells from the cell suspension, as these cells attach to an uncoated culture dish while neurons remain suspended. Thus, primary hypothalamic cells were plated onto an uncoated petri dish and incubated for 30 minutes at 37°C in 5% CO_2 and 95% air. The suspension was collected and pelleted by centrifugation (5,000 g for 5 minutes). Primary hypothalamic neurons were re-suspended in phenol-red free SCM medium, plated onto culture dishes or coverslips coated with poly-D-Lysine (Sigma) at a density of 2,500 cells/mm^2, and incubated at 37°C in 5% CO_2 and 95% air. On day 2 medium was changed to SCM free of L-glutamine. On day 4 medium was changed to serum-free medium (SFM) that consisted of phenol-red free neurobasal medium containing 1% N2 supplement, 15 mM Hepes, 1.5 mM sodium pyruvate, 30 μg/mL penicillin-streptomycin, and 10 μM of the anti-mitotic agent cytosine arabinoside (Sigma). Half of the medium was then changed every other day and cultures were maintained for 8 or 9 days *in vitro* (DIV). Culture materials were purchased from Invitrogen unless otherwise stated.

2.8.2 Pharmacological Agents

The pharmacological agents used in this study included 17β-estradiol (E2, Sigma-Aldrich); the selective ERβ agonist, diarylpropionitrile (DPN, Tocris); the Src inhibitor, PP2 (Calbiochem, San Diego); the inactive analog of PP2, PP3 (Calbiochem); the PI3K inhibitor, LY294002 (Cayman Chemical, Ann Arbor); and the MAPK inhibitor, PD98059 (Cayman Chemical). All pharmacological agents were freshly diluted in neurobasal medium from stock solutions prepared in ethanol or DMSO; final ethanol or DMSO concentrations were less than 0.001%.

2.8.3 Antibodies

The following primary antibodies were used for immunocytochemistry: mouse anti-microtubule associated protein-2 (MAP-2, 1:100, GeneTex, San Antonio, TX), rabbit anti-glial fibrillary associated protein (GFAP, 1:100, Abcam, Cambridge, MA), rabbit anti-nNOS (1:500, Santa Cruz, Santa Cruz, CA), and chicken anti-ERβ 503 IgY (1:100, kindly provided by J.A. Gustafsson (Huddinge, Sweden). The latter antibody has been used extensively to detect ERβ immunoreactivity in rat tissue [220-222]. The following primary antibodies were used for western blot analysis: rabbit anti-phospho-nNOS (1:500, Abcam) which recognizes rat nNOS phosphorylated at Ser^{1412}, rabbit anti-nNOS (1:500, Santa Cruz, Santa Cruz, CA), rabbit anti-phospho-Src at Tyr^{416} (1:1000, Cell Signaling, Danvers, MA), rabbit anti-Src (1:3000, Santa Cruz), mouse anti-β-actin (1:4000, Sigma), rabbit anti-phospho-Akt at Ser^{473} (1:500, Cell Signaling), rabbit anti-Akt (1:1000, Cell Signaling), rabbit anti-phospho-ERK1/2 (1:2000, Cell Signaling), and rabbit anti-ERK1 (Santa Cruz).

2.8.4 Immunocytochemistry of hypothalamic neurons

Primary hypothalamic neuronal cultures grown on coverslips for 8-9 days in vitro (DIV) were rinsed in PBS and fixed for 15 minutes in 3% paraformaldehyde. Free aldehydes were quenched with NH_4Cl buffer for 10 minutes; neurons were permeablized in 0.1% Triton-X 100 for 4 minutes and incubated in blocking solution (PBS containing 0.1% Tween 20 and 0.5% nonfat dry milk) for 30 minutes. To immunohistochemically define the composition of the cultures, hypothalamic neuronal cultures were incubated in anti-MAP-2, a neuronal marker, and anti-GFAP, an astrocyte marker, for 2 hours at room temperature followed by a 1 hour incubation in anti-rabbit Alexa fluor 488 and anti-mouse Alexa fluor 647 (Invitrogen). For double-labeling with ERβ and nNOS, cultures were incubated in anti-ERβ for 72 hours at 4^oC, and then incubated for 1 hour in anti-chicken rhodamine (Jackson Immunoresearch). Cultures were then incubated again in blocking solution for 30 minutes followed by incubation in anti-nNOS for 2 hours at room temperature. Finally, cultures were incubated in anti-rabbit Alexa fluor 488 (Invitrogen) for 1 hour before being rinsed in PBS and mounted on slides using Vectashield mounting medium (Vector Laboratories) containing 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei.

2.8.5 Western blot analysis

Primary hypothalamic neurons were harvested following treatment with various pharmacological agents and homogenized in lysis buffer (0.01 m PBS, 0.1% tergitol, 0.5% sodium deoxycholate) containing 0.1% sodium dodecyl sulfate, 0.5 mm sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonylfluoride, 10 μg/ml aprotinin and 1:100 protein phosphatases inhibitor cocktail 1. Proteins were quantified using the Bradford assay (BioRad Laboratories) to ensure equal loading into the gel. Proteins were separated by electrophoresis on an 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (containing 0.05% Tween 20) at 4°C and incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. Membranes were incubated in secondary antibodies conjugated to horseradish-peroxidase for 1 hour at room temperature. Immunocomplexes were visualized using a chemiluminescence reagent (PerkinElmer, Boston, MA) and autoradiography film. Mean protein densities were measured using Image J software (NIH). Changes in levels of protein expression were determined by calculating the ratio of phospho-protein density to total protein density.

2.8.6 Quantification of NO release

NO production was determined by measuring the accumulation of nitrite, a metabolite of NO, in the culture medium using the 2,3 diaminonaphthalene (DAN) fluorometric assay as previously described [121]. Briefly, medium from primary hypothalamic neurons (8-9 DIV) treated with various pharmacological agents was collected and incubated with 40 uL of DAN (50 μg/mL in 0.62M HCl) for 15 minutes. The reaction was terminated with 50 μL of 2.8M NaOH. Fluorescence was measured using a spectrofluorometer (QM-4SE, Photon Technology International, Lawerenceville, NJ) with excitation of 365 nm and emission of 450 nm and the amount of nitrite was determined by comparison to a standard curve with $NaNO_2$.

2.9 Statistical analyses

Statistical analysis was carried out using SigmaStat software. All data are presented as means ± SEM. Significance was determined using either one-way or two-way ANOVA. If determined by one-way ANOVA that differences in means among the treatment groups were statistically different (when $p<0.05$) or if determined by a two-way ANOVA that there was a statistically significant interaction between groups (when $p<0.05$), a pairwise multiple comparison was performed using a post hoc test, indicated below. The post hoc test following either a one-way or two-way ANOVA further determined that differences between specific treatment groups were statistically significant when $p<0.05$.

2.9.1 Hypothalamic slice cultures

Each measurement was taken from one hypothalamic slice obtained from a different animal and repeated in two to three independent experiments $N = 6 - 14$. Significant differences were determined using either one-way or two-way ANOVA followed by the post hoc Student-Newman Keuls Method or Tukey test.

2.9.2 Microinjections into the PVN

Data for each group was obtained from $N \geq 6$ animals. Significant differences were determined by one-way or two-way ANOVA followed by the post hoc Tukey test.

2.9.3 Primary hypothalamic cultures

Data were obtained from at least 4 independent experiments. Significant differences were determined using either one-way or two-way ANOVA followed by the post hoc Tukey test.

Chapter 3

Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an ERβ-dependent mechanism in hypothalamic slice cultures

A version of this chapter has been published as "Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an ERβ-dependent mechanism in hypothalamic slice cultures" (Sarah Gingerich and Teresa L. Krukoff. 2005. Endocrinology 146:2933-2941). Reprinted with permission.

3.1 Introduction

Hormone replacement therapy (HRT) is widely used in women to relieve menopausal symptoms and, until recently, was believed to protect aging women from cardiovascular disease. However, the 2002 Women's Health Initiative study on HRT created a great deal of controversy when it was halted abruptly because of risks associated with HRT, including increased incidence of stroke [223, 224]. These findings dramatically demonstrate that, not only are the effects of HRT in women poorly understood, but also that estrogen's effects on normal physiological processes are still unclear.

We recently reported that ovariectomized E2-treated (OVX-E) rats display attenuated blood pressure (BP) responses to psychological stress compared to OVX vehicle-treated (OVX-V) rats [86]. In agreement with our results, peri- and post-menopausal women receiving E2 replacement therapy exhibit lower BP responses to mental stress compared to those not receiving E2 [85, 225]. A potential mediator of E2's effect on cardiovascular responses to stress is nitric oxide (NO), a neurotransmitter involved in maintaining homeostasis and regulating autonomic activity [150, 152]. Indeed, we found that E2 acts through brain NO to attenuate BP responses to psychological stress [86].

Studies performed in both peripheral and brain tissues have demonstrated that E2 influences the NO system. For example E2 increases NO production [186, 226], endothelial nitric oxide synthase (eNOS) expression [186, 227], and eNOS activity [186, 226] in cultured endothelial cells. E2 also increases eNOS protein expression in cerebral microvessels [178, 180, 202, 203]. Finally, E2 increases NO production [199, 201] and eNOS protein expression [204] in *ex vivo* preparations of the median eminence. The effects of E2 on nNOS in the brain appear to be regionally specific. E2 has been shown to increase the numbers of NADPH-diaphorase (neuronal NOS [nNOS]) neurons [193, 194] and nNOS mRNA [196, 197] in select brain regions of OVX rats. Others report that E2 does not change nNOS mRNA levels or numbers of nNOS neurons in other brain regions [196, 228]. The specific mechanism(s) through which E2 acts on NO in the brain remains to be elucidated.

As the paraventricular nucleus (PVN) of the hypothalamus is an autonomic center which regulates neuroendocrine and autonomic functions [152], we hypothesize that E2 acts on the NO system in the PVN to alter central autonomic activity. Since little is known about how E2 affects the NO system in the PVN, our first aim was to investigate E2's effects on NOS expression in the PVN. Because E2 acts on two receptors, estrogen receptor-α (ERα) and ERβ, and the PVN expresses only ERβ [94, 96, 100, 217], our second aim was to investigate the role of ERβ in E2's effects on NOS expression in the PVN. Using hypothalamic slice cultures, we investigated the effects of E2 on eNOS and nNOS expression in the PVN. We used the ER antagonist, ICI 182,780, to determine if E2 affects NOS expression through an ER-dependent pathway. We also used the selective ERβ agonist, genistein, the selective ERα agonist, propyl pyrazole triol (PPT), and the selective ERα antagonist, methyl-piperidino-pyrazole (MPP), to determine if E2 acts through an ERβ-dependent mechanism to affect NOS expression.

3.2 Results

3.2.1 eNOS and nNOS are expressed in the PVN of hypothalamic slice cultures

eNOS protein expression was seen throughout the slices in blood vessel-like structures. The PVN displayed a higher density of eNOS-IR vessels compared to surrounding areas (Fig. 3-1A). These positively stained structures were verified to be blood vessels based on double-labelling for eNOS and RECA-1, a marker for endothelial cells [214] (Figs. 3-1E, F, G). Most nNOS-IR neurons were localized in the PVN (Fig. 3-1B). Untreated hypothalamic slice cultures did not express iNOS (Fig. 1C). As others have shown that LPS treatment induces iNOS expression in hippocampal slice cultures [229, 230], LPS treatment was used as a positive control. Hypothalamic slice cultures treated with LPS (1 μg/mL) for 24 hours expressed iNOS within the PVN (Fig. 3-1D).

3.2.2 ERβ is expressed in the PVN

ERβ-positive neurons were found in the PVN of hypothalamic slices (Fig. 3-1H). ERα mRNA was not expressed in the PVN (Fig. 3-1I), but was found in the ventral medial hypothalamus (VMH) (Fig. 3-1J).

3.2.3 E2 alters NOS expression

Slice cultures treated with E2 (0.1 nM – 10 nM) for 48 hours displayed a dose-dependent increase in eNOS protein expression in the PVN, with a 36% increase when treated with 1 nM E2, compared to control cultures (Figs. 3-2A, 3-2B, and 3-3A). E2 at 10 nM did not change eNOS expression compared to controls. All concentrations of E2 significantly decreased the numbers of nNOS-positive neurons in the PVN. E2 at 1 nM decreased the numbers of nNOS-IR neurons by 28% compared to control slice cultures (Figs. 3-2D, 3-2E, and 3-3B)

Based on dose response data, 1 nM E2 was used in all subsequent experiments, as it was the lowest concentration that significantly altered eNOS and nNOS protein expression. Compared to time-matched controls, 1 nM E2 significantly increased eNOS expression in the PVN at 8, 24, and 48 hours (31%, 34% and 41%, respectively) (Fig. 3-4A). E2 decreased the numbers of nNOS-IR neurons in the PVN at 24 and 48 hours (23% and 32%, respectively) compared to time-matched control cultures (Fig. 3-4C).

3.2.4 Genistein alters NOS expression

Slice cultures treated with genistein (10 nM –10 μM, 48 hours) displayed a dose-dependent increase in eNOS expression with an increase of 42% when treated with 0.1 μM genistein compared to control cultures (Fig. 3-2A, 3-2C, and 3-3C). The numbers of nNOS-IR neurons were decreased by approximately 28% at all concentrations of genistein compared to controls (Fig. 3-2D, 3-2F, and 3-3D).

Based on the dose response data, 0.1 μM genistein was used in all subsequent experiments, as it was the lowest concentration that significantly altered eNOS and nNOS protein expression. Compared to time-matched controls, 0.1 μM genistein significantly increased eNOS expression at 8, 24, and 48 hours (21%, 32%, and 31%, respectively) (Fig. 3-4B). Genistein decreased the numbers of nNOS-IR neurons in the PVN at 8, 24 and 48 hours (27%, 28%, and 22%, respectively) compared to time-matched controls (Fig. 3-4D).

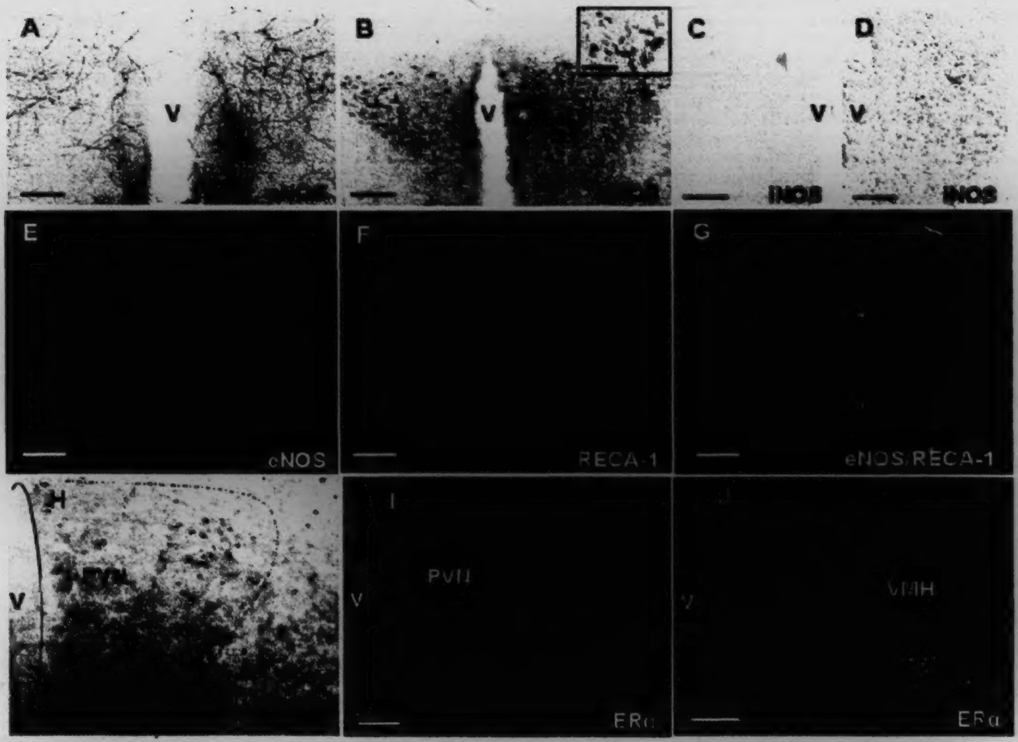

Figure 3-1. Representative photographs of the PVN in control hypothalamic slice cultures (A-C, E-I) and in a culture treated with lipopolysaccharide (LPS, D). (A) eNOS expression in blood vessel-like structures; (B) nNOS-positive neurons. Inset in B shows a higher magnification of the outlined region. (C) lack of iNOS expression in control slices; (D) iNOS expression in a LPS-treated slice (1 μM, 24 hours). (E) eNOS expression; (F) expression of RECA-1, a marker for endothelial cells. (G) merged image showing that blood vessels are double-labeled with eNOS and RECA-1. (H) ERβ expression (immunohistochemistry) in the PVN. (I) lack of ERα mRNA (*in situ* hybridization) in the PVN. (J) ERα mRNA in the ventral medial hypothalamus (VMH). V = third ventricle. Scale bars: A-D = 200 μm; inset in B = 100 μm; E-G = 50 μm; H, I and J = 100 μm.

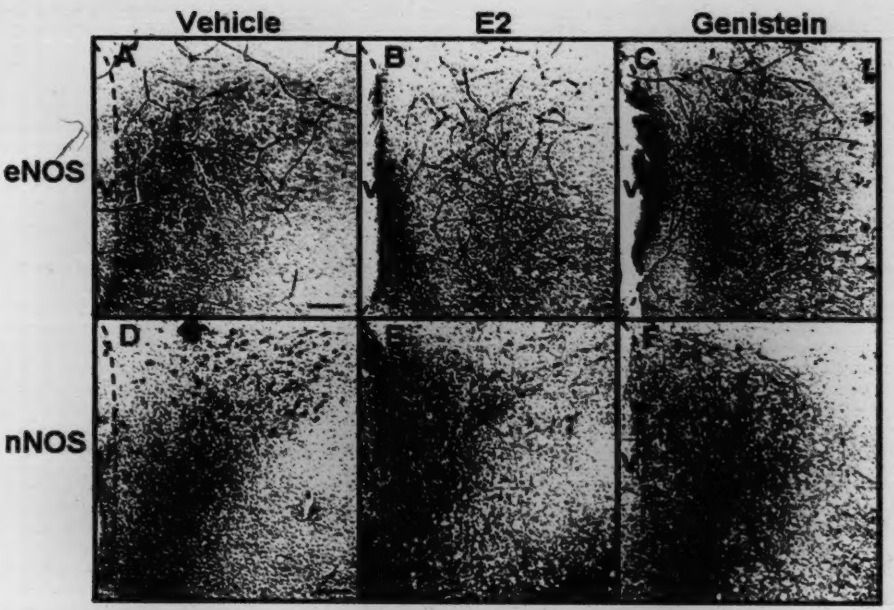
Vehicle
E2
Genistein
A
B
C
D
E
F
eNOS
nNOS
V
V
V
V
V

Figure 3-2. Representative photographs of eNOS (A-C) and nNOS (D-F) expression in the PVN of hypothalamic slice cultures treated with vehicle (A, D), 1 nM E2 (B, E), or 0.1 μM genistein (C, F) for 48 hours. V= third ventricle. Scale bar = 100 μm.

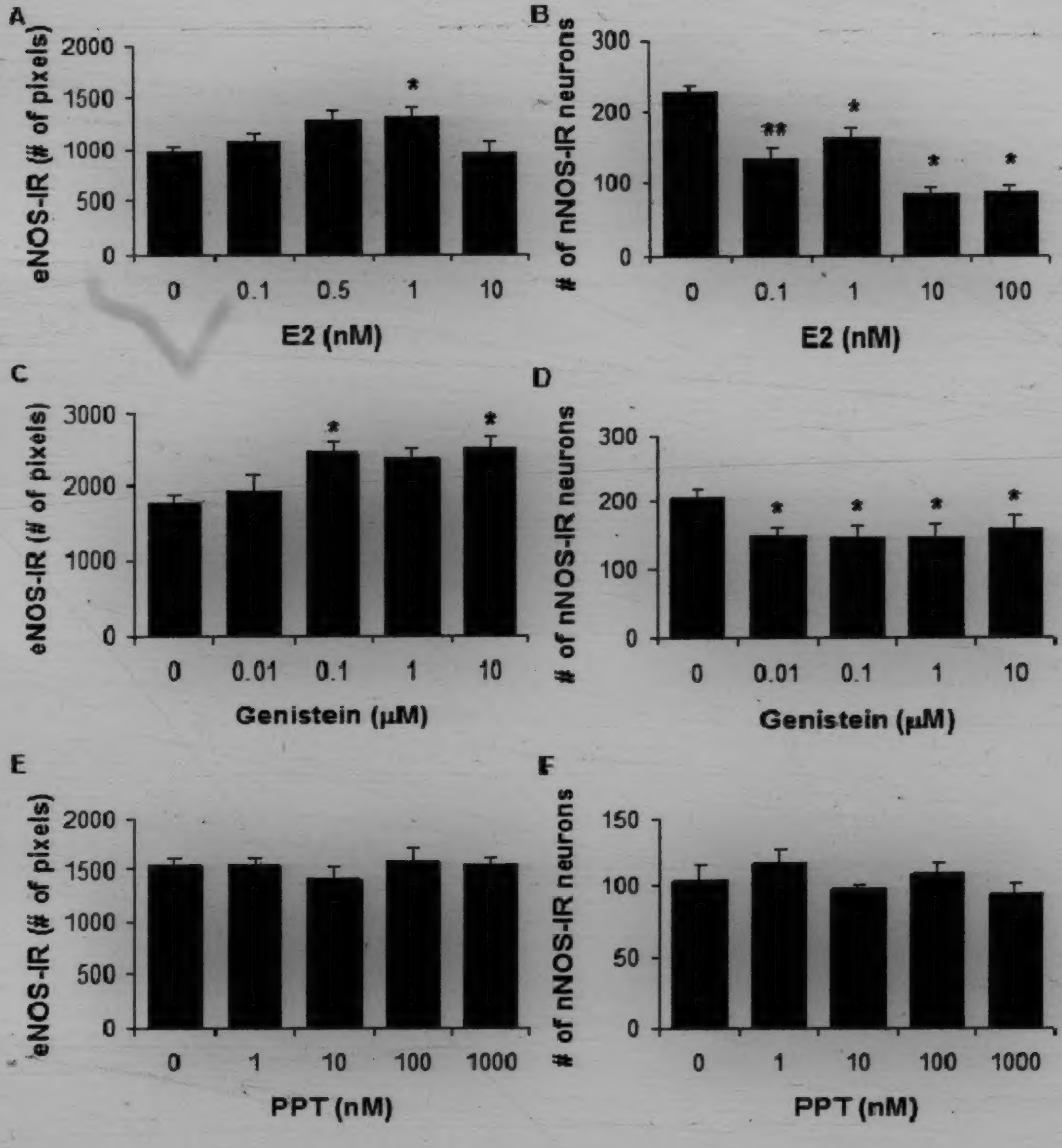

Figure 3-3. Dose-response effects of E2 (A, B), genistein (C, D), and PPT (E, F) on eNOS (A, C, E) and nNOS (B, D, F) expression in the PVN of hypothalamic slice cultures. Cultures were treated with E2 (0.1 nM – 10 nM), genistein (0.01 μM – 10 μM) or PPT (1 nM – 1000 nM) for 48 hours. Results are expressed as means ± SEM; n≥6 for each group. * $p<0.05$, ** $p<0.001$ versus control.

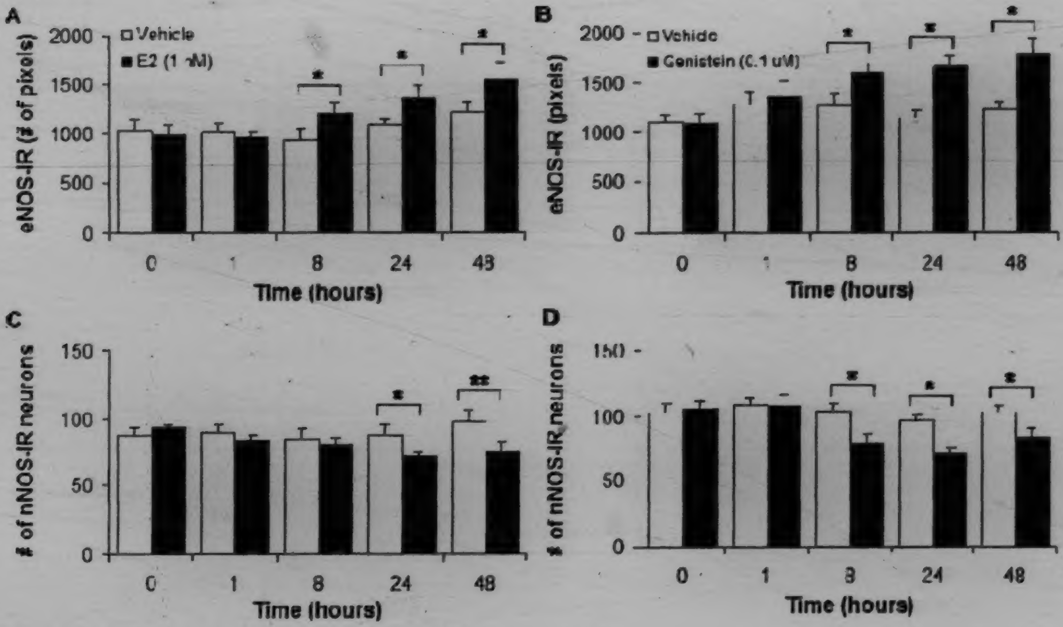

Figure 3-4. Time-course effects of E2 (A, C) and genistein (B, D) on eNOS-IR (A, B) and numbers of nNOS-IR neurons (C, D). Cultures were treated with E2 (1 nM) or genistein (0.1 μM) for 0, 1, 8, 24, or 48 hours. Results are expressed as means ± SEM; n≥6 for each group. * $p<0.05$, ** $p<0.001$ versus time-matched control.

3.2.5 E2 and genistein alter NOS expression equally in the parvo- and magnocellular regions of the PVN

E2 and genistein altered eNOS and nNOS expression in the parvocellular and magnocellular regions of the PVN. However, E2 and genistein treatments did not change eNOS or nNOS expression in the parvocellular and magnocellular regions as a proportion of total expression in the entire PVN (Table 3-1). In all subsequent experiments, therefore, the PVN was analyzed as a single entity.

3.2.6 E2 and genistein alter eNOS expression exclusively in the PVN of slice cultures

Compared to their vehicle-treated controls, neither E2 (1 nM) nor genistein (0.1 μM) treatment for 24 hours affected eNOS expression in an area adjacent to the PVN; E2: 654 ± 20 pixels vs. 665 ± 14 (controls); genistein: 662 ± 28 pixels vs. 676 ± 22 (controls).

3.2.7 Effects of E2 and genistein on NOS expression are ER-dependent

To determine if the changes in eNOS and nNOS expression in the PVN stimulated by E2 or genistein are mediated by an ER-dependent mechanism, E2 (1nM) or genistein (0.1 μM) was applied to slice cultures for 24 hours in the presence or absence of the non-selective ER antagonist, ICI 182,780. ICI 182,780 (10 nM) blocked the E2-induced effects on eNOS expression and on numbers of nNOS-IR neurons in the PVN (Fig. 3-5A, C). All concentrations of ICI 182,780 (1 nM -1 μM) blocked the genistein-induced effects on eNOS and nNOS expression in the PVN; Figures 3-5B and 3-5D illustrate the results for ICI 182,780 at 10 nM.

3.2.8 Effects of E2 and genistein are not ERα-dependent

To determine if the increases in eNOS expression in the PVN stimulated by E2 and genistein are mediated by an ERα-dependent mechanism, E2 or genistein was applied to cultures for 24 hours in the presence or absence of the selective ERα antagonist, MPP (100 nM – 10 μM). None of the concentrations of MPP used altered the effects of E2 or genistein on eNOS expression; Figure 3-6 shows the results for MPP at 1 μM.

3.3 Discussion

In the present study we investigated the effects of E2 on the NO system in hypothalamic slice cultures. We show that E2 increases eNOS expression and decreases the numbers of nNOS-positive neurons in the PVN. Using a selective ERβ agonist, a selective ERα agonist, and a selective ERα antagonist, we show that E2-induced changes in NOS expression in the PVN of hypothalamic slice cultures are ERβ-dependent.

Little is known about how E2 affects the NO system in autonomic centers of the brain, and in particular, the PVN. *In vivo* studies are often difficult to interpret due to physiological influences from other brain regions. For this reason, we used hypothalamic slice cultures to investigate E2's effect on the NO system as this model eliminates influences from other brain regions, and maintains the cellular morphology and organization of the hypothalamus [231, 232]. Although the hypothalamic tissues used in these cultures were obtained from postnatal rats, a comprehensive study that investigated expression of ERs in postnatal rat brain (P3, 7 and 14) revealed that, with the exception of the arcuate nucleus and the supramammillary complex, postnatal and adult

Table 3-1. Effects of E2 (1 nM) and genistein (0.1 µM) treatment (24 hours) on NOS expression in the parvocellular and magnocellular regions of the PVN as a proportion of NOS expression in the entire PVN.

Treatment	NOS isoform	% of total expression Parvocellular region		% of total expression Magnocellular region	
		Control	Treated	Control	Treated
E2	eNOS	51 ± 4	54 ± 2 n.s.	49 ± 4	46 ± 2 n.s.
E2	nNOS	58 ± 2	57 ± 3 n.s.	42 ± 2	43 ± 3 n.s.
Genistein	eNOS	54 ± 4	57 ± 2 n.s.	46 ± 4	43 ± 2 n.s
Genistein	nNOS	51 ± 2	54 ± 2 n.s.	49 ± 2	46 ± 2 n.s.

Mean ± SEM, $n \geq 6$, n.s. = no significant difference.

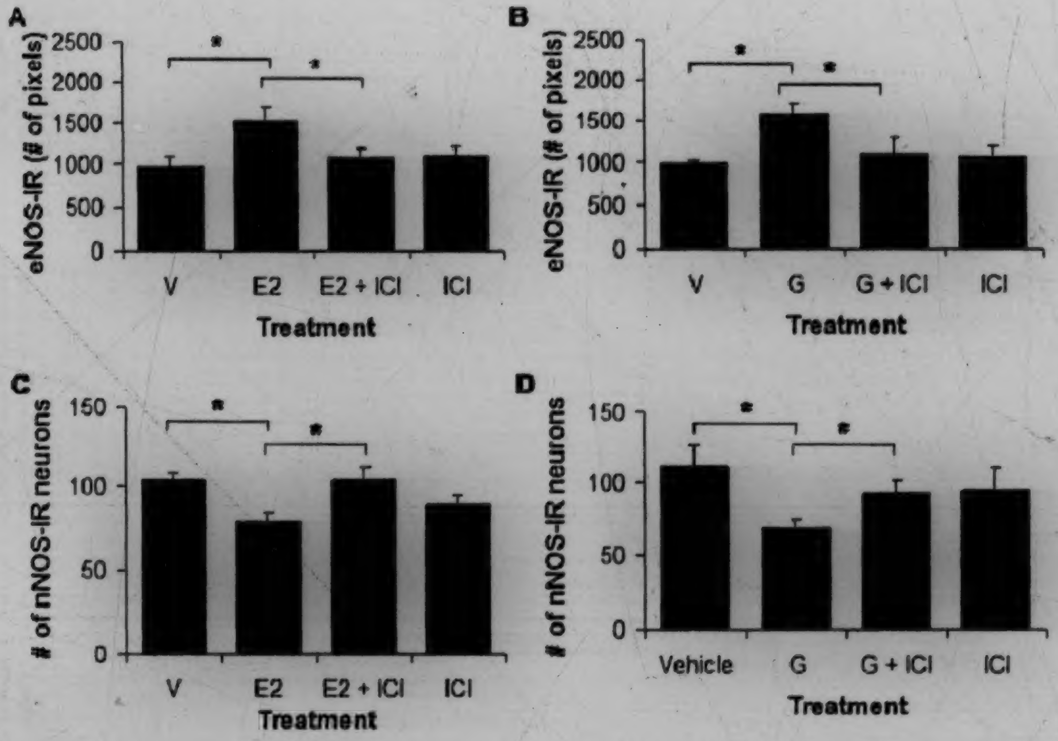

Figure 3-5. Effects of ICI 182,780 on E2-induced (A, C) and genistein-induced (B, D) changes in eNOS (A, B) and nNOS (C, D) expression in the PVN of hypothalamic slice cultures. Cultures were treated with E2 (1 nM) or genistein (0.1 μM) for 24 hours in the presence or absence of ICI 182,780 (10 nM). Results are expressed as means ± SEM; n≥8 for each group. * $p<0.05$.

hypothalamic tissues exhibit the same expression patterns of ERs [233]. Furthermore, we show that ERβ (but not ERα) is expressed in the PVN from hypothalamic slice cultures, as is the case in adult hypothalamus *in vivo*. Finally, we also show that, like the hypothalamus *in vivo*, hypothalamic slice cultures express basal levels of eNOS and nNOS but not iNOS. Therefore, based on these data, we conclude that hypothalamic slice cultures constitute an appropriate model for investigating the effects of E2 on the NO system of the PVN.

We demonstrate that E2 stimulates increased eNOS expression in the PVN of hypothalamic slices in a time-dependent manner. These results agree with other studies which have shown that E2 stimulates eNOS expression and activity in cultured endothelial cells of the aorta and umbilical cord [186, 226, 227, 234]. Furthermore, E2 treatment in ovariectomized (OVX) rats has been shown to stimulate eNOS protein expression in cerebral microvessel fractions and isolated pial arterioles [178, 180, 202, 203] and in *ex vivo* preparations of the median eminence [204]. Our data indicate that the E2-stimulated increases in eNOS expression are in fact due to increases in the density of eNOS-IR blood vessels, suggesting that E2 promotes angiogenesis in the PVN. E2 treatment has also been shown to stimulate angiogenesis in OVX mice, demonstrated by increased vascularization of a subdural gel plug [22]. Interestingly, we show that, within our hypothalamic slice cultures, E2 appears to stimulate eNOS expression exclusively within the PVN. This finding emphasizes the importance of this nucleus as a target for E2's effects on eNOS expression in the hypothalamus. Finally, we found that the effect of E2 on eNOS expression is concentration-dependent as

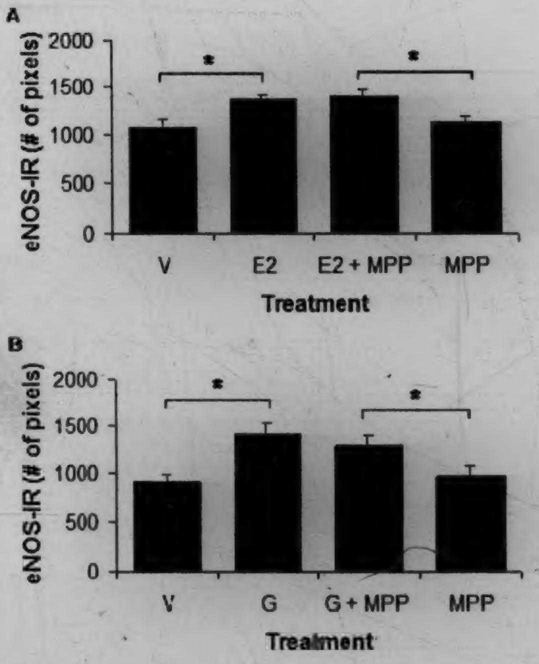

Figure 3-6. Effects of MPP on E2-induced (A) and genistein-induced (B) changes in eNOS-IR in hypothalamic slice cultures. Cultures were treated with either E2 (1 nM) or genistein (0.1 μM) for 24 hours in the presence or absence of MPP (1 μM). Results are expressed as means ± SEM; n≥7 for each group. * $p<0.05$.

10 nM E2 did not alter eNOS expression. The reasons for this result is not known, but it is possible that 10 nM E2 treatment has adverse effects on the hypothalamic tissue as it has been shown that E2 at this concentration induces oxidative DNA damage in a variety of cells and tissues including, MCF-7 breast cancer cells, kidney, and liver tissue [235, 236].

We show that E2 decreases the numbers of nNOS-positive neurons in the PVN *in vitro*. *In vivo* studies have shown that E2 increases levels of nNOS mRNA in the ventral medial nucleus [196] and the hippocampus [197], but reports on E2's effect on nNOS expression in the PVN are conflicting. Some groups report no changes in nNOS mRNA levels [196] or numbers of NADPH-diaphorase neurons [196, 228] in the PVN of OVX-E rats, while another group reported that OVX decreases the numbers of NADPH-diaphorase neurons in the PVN and that E2 treatment reverses this effect [195]. Finally, E2 decreases levels of nNOS protein expression in nerves and ganglia of the vagina and clitoris of rabbits [237]. The significance of these divergent findings is unclear at this time, but they may be due to the fact that different parameters were measured in each of the studies.

Multiple pathways of estrogen signalling have been described; estrogen mediates genomic or non-genomic effects through receptor-dependent and -independent pathways [234, 238-241]. ER-dependent genomic mechanisms involve binding of an estrogen-ER complex to the estrogen response element of the target gene promoter to regulate nuclear transcription [234, 238], while ER-dependent non-genomic mechanisms involve membrane ERs and rapid activation of signalling cascades such as mitogen-activated protein and protein kinase C pathways [140, 234, 238, 242, 243]. ER-independent mechanisms appear to include "non classical" membrane receptors which act on intracellular signalling pathways and are not blocked by the non-selective ER antagonist, ICI 182,780 [243]. Our data show that E2 stimulates increased eNOS expression after 8 hours, suggesting that E2 acts through a slow, genomic pathway to alter transcription of the eNOS gene. Indeed, the eNOS promoter contains an estrogen response element [182]. Our data also show that E2-induced changes in NOS expression are ER-dependent as ICI 182,780, used at concentrations (1 nM- 1μM) shown by others to effectively block ERs [121, 202], inhibits the effects of E2 on eNOS and nNOS. Our findings are consistent with other studies which show that E2 increases eNOS expression by a genomic ER-dependent mechanism in isolated cerebral vessels from adult rats [202]. We and others have reported non-genomic or membrane ER-dependent up-regulation of eNOS activity and/or NO production by E2 in cultured uterine endothelial cells [183], neuroblastoma cells [121], and pedal ganglia of molluscs [244].

Due to homologous regions in the genes for ERα and ERβ [94, 96], few selective ligands for each receptor exist and to date there are no selective ERβ antagonists commercially available. Therefore, to study activation of ERβ, we used genistein as it confers estrogenic actions at concentrations between 1 nM and 1 μM [208, 245], is 20 times more selective for ERβ than ERα [207, 208, 245, 246], and does not activate ERα-dependent gene expression in the hypothalamus *in vivo* [207]. We did not use higher concentrations of genistein (upper μM range), as they inhibit protein tyrosine kinases [247, 248]. Our data show that genistein increases eNOS expression in the PVN and decreases the numbers of nNOS-positive neurons in the PVN at all concentrations used. In agreement with our results, E2 increased eNOS-promoter activity in cardiac myocytes and this effect was blocked with a selective ERβ antagonist developed by this group [249]. These results support our hypothesis that ERβ mediates the E2-induced changes in NOS expression in the PVN.

While neurons of the PVN express ERβ and no ERα [96, 100, 217], cerebral blood vessels express both ERβ and ERα in a ratio of 3:1 [191]. Therefore, to investigate the possibility that activation of ERα alters NOS expression, we used the highly selective ERα agonist, PPT [209, 250]. Our data show that activation of ERα has no effect on either eNOS or nNOS expression in the PVN. We also used the selective ERα antagonist, MPP [209, 211], to exclude the possibility that ERα contributes to the E2-induced increase in eNOS expression. Our data show that ERα is not involved in the E2- or genistein-induced increases in eNOS expression in hypothalamic slice cultures. Interestingly, OVX-E ERα-knockout mice apparently do not display altered levels of eNOS expression in cerebral microvessels compared to OVX-V ERα-knockout mice [251]. However, this study did not account for the finding that ERα-knockout mice expressed higher levels of eNOS compared to wild type mice. Furthermore, developmental differences between ERα-knockout mice and wild-type mice may also contribute to the differences between the results of this study and ours.

In conclusion, we show that E2 stimulates increases in eNOS protein expression in the PVN and decreases the numbers of nNOS-positive neurons in the PVN of hypothalamic slice cultures. Furthermore, our time-course data suggest that E2 alters NOS expression via a slow, genomic effect. Finally, our morphological data in combination with our use of a variety of selective ER agonists and antagonists provide strong evidence that ERβ is responsible for E2's effect on NOS expression in the PVN. We previously showed that E2 treatment attenuates blood pressure responses to psychological stress in OVX rats through brain NO produced by eNOS and/or nNOS [86]. Therefore, our current results raise the intriguing possibility that NO produced by eNOS in blood vessels of the PVN via an ERβ-dependent mechanism acts on neighbouring neurons to influence autonomic pathways originating from the PVN.

Chapter 4

Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor β and NO

A version of this chapter has been published as "Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor β and NO" (Sarah Gingerich and Teresa L. Krukoff. 2006. Hypertension. 48:1130-1136). Reprinted with permission.

4.1 Introduction

The increased incidence of cardiovascular disease in postmenopausal women has been attributed to the loss of estrogen [252, 253] and it was observed that hormone replacement therapy (HRT) confers cardiovascular benefits in these women [252-254]. In 1993 the Women's Health Initiative (WHI) trial began recruiting over 16,000 postmenopausal women to assess the benefits and risks of HRT on the incidence of heart disease, cancers, and fractures [223]. Surprisingly, the trial was terminated in 2002 due to increased risks of breast cancer and stroke [223]. While the WHI trial has been criticized for its design and conclusions [253, 255], the trial illustrates the need to better understand estrogen's effects on the cardiovascular system and on general physiology, in order to develop specific therapies that will contribute to cardiovascular health in women and men.

17β-estradiol (E2) provides cardiovascular benefits in response to stress. E2 decreases sympathetic activity [67] and blood pressure (BP) responses to mental stress in postmenopausal women [83, 85]. We and others have shown that E2 attenuates BP and heart rate (HR) responses to restraint stress [86, 87] and cage switch [87] in ovariectomized (OVX) rats. E2 in the brain also modulates cardiovascular function, as microinjections of E2 into the parabrachial nucleus (PBN) [256] and the nucleus of the solitary tract (NTS) [78] of male rats decrease resting BP and HR and increase baroreceptor sensitivity. These data demonstrate that E2 modulates cardiovascular responses to stress and that the brain is an important site of E2's action.

The paraventricular nucleus (PVN) of the hypothalamus regulates neuroendocrine and autonomic functions [257]. E2 attenuates the numbers of activated neurons in the

PVN of OVX rats induced by footshock [80] and immobilization [258], and blocking endogenous E2 in the PVN decreases the amount of restraint stress-induced corticosterone release in cycling female rats [259]. These data suggest that E2 acts in the PVN to affect homeostatic processes, but the effects of E2 in the PVN on cardiovascular function have not been investigated.

Nitric oxide (NO) is an important regulator of sympathetic activity. NO in the PVN tonically inhibits sympathetic output to the periphery and inhibition of NO release here increases sympathetic output [150, 175, 260]. We have shown that NO in the brain mediates E2's effect on BP responses to restraint stress in OVX rats [86]. Furthermore, NO levels increase in response to restraint/immobilization stress in the hypothalamus and brainstem of E2-treated OVX rats [86] and in the PVN of E2-treated male rats [177]. E2 can act on NO in the PVN, as neurons here express neuronal nitric oxide synthase (nNOS) and estrogen receptor β (ERβ) [217, 220] and cerebral blood vessels express endothelial NOS (eNOS) and both ERα and ERβ [191]. We have recently shown that, in the PVN of rat hypothalamic slice cultures, E2 alters the expression of nNOS and eNOS via ERβ [220]. These results revealed a relationship between E2 and NO in the PVN, but the functional significance of this relationship is unknown. We hypothesize that E2 affects NO release in the PVN to modulate cardiovascular function.

γ-aminobutyric acid (GABA) also plays an important role in regulating autonomic activity. In the PVN, GABA inhibits sympathetic output [175, 261] and mediates the inhibition of sympathetic output by NO [175, 212]. GABA has also been shown to mediate E2's effects on cardiovascular function in the PBN [256]. These studies suggest that GABA may also mediate E2's cardiovascular effects in the PVN.

The brain is an important, yet often overlooked, target of E2's actions on cardiovascular function. While the cardiovascular effects of E2 in a small number of brainstem autonomic nuclei have been studied [78, 256], the functional effects of E2 in the PVN are unknown. The goals of this study were to investigate the acute effects of E2 microinjections into the PVN on resting BP and HR and on L-glutamate-induced increases in BP and HR. We also studied the roles of ERs in E2's effects using agents selective for ERα and ERβ. Finally, we investigated the roles of NO and GABA in E2's effects using agents selective for nNOS, eNOS, and the $GABA_A$ receptor.

4.2 Results

4.2.1 Microinjection of E2 into the PVN attenuates the L-glutamate-induced pressor response

Microinjections of vehicle or E2 (0.1 – 10 pmol, 100 nL) into the PVN had no effects on baseline MAP (83 ± 2 mm Hg) or HR (375 ± 5 beats/min) (data not shown). Microinjections of L-glutamate (50 nmol, 100 nL) 30 minutes following microinjection of saline significantly increased MAP and HR by 14 ± 3 mm Hg and 30 ± 6 beats/min (Figs. 4-1A, C, E). L-glutamate also increased the AUC for the MAP and HR responses (71 ± 34 mm Hg x S and 146 ± 68 beats/min x S; Figs. 4-1D, F). Microinjections of E2 (0.1, 1, and 10 pmol) into the PVN 30 minutes prior to L-glutamate dose-dependently attenuated the increases in MAP by up to 59% with 10 pmol E2. The AUC for the MAP response was also attenuated with 10 pmol E2 (from 71 ± 34 for saline to -39 ± 15 mm

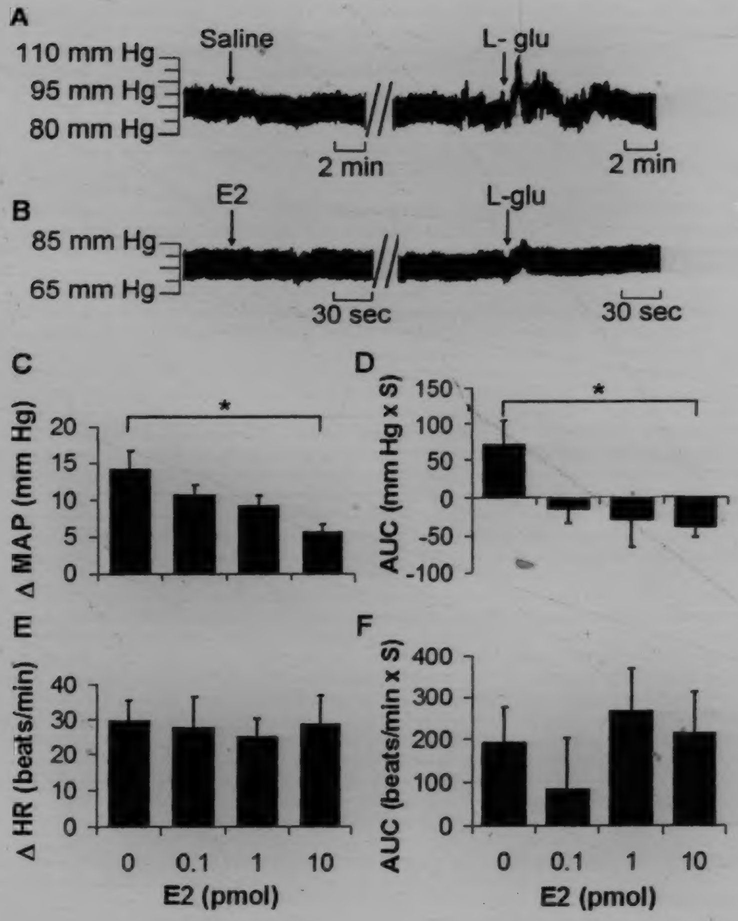

Figure 4-1. Representative MAP traces following injection of (A) saline or (B) E2 (10 pmol) into the PVN followed by L-glutamate (L-glu) 30 minutes later. Effect of L-glutamate injection into the PVN following E2 injection (0 – 10 pmol) on (C) the change in MAP, (D) AUC for the MAP response, (E) change in HR, and (F) AUC for the HR response. Data represents mean ± SEM. n≥ 8. * = $p<0.05$.

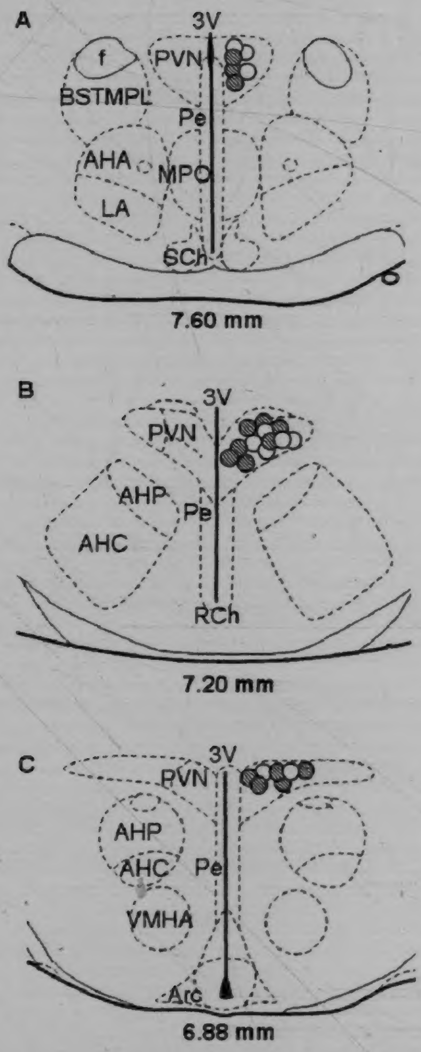

Figure 4-2. (A – C) Schematic representations of serial coronal sections from 6.88 mm to 7.60 mm anterior to interaural zero showing the injection sites of 25 rats. Each hatched circle represents the injection site of a saline-treated rat n=15 and each open circle represents the injection site of an E2 (10 pmol)-treated rat n=10. 3V, third ventricle; AHA, anterior hypothalamic area, anterior part; AHC, anterior hypothalamic area, central part; AHP, anterior hypothalamic area, posterior part; Arc, arcuate nucleus; BSTMPL, bed nucleus of the stria terminalis, medial division, posterolateral part; f, fornix; LA, lateroanterior hypothalamic nucleus; MPO, medial preoptic nucleus; Pe, periventricular hypothalamic nucleus; PVN, paraventricular hypothalamic nucleus; RCh, retrochiasmatic area; SCh, suprachiasmatic nucleus; VMHA, ventromedial hypothalamic nucleus, anterior part. This drawing was modified from Paxinos and Watson [262].

Hg x S) (Figs. 4-1B, C, D). E2 had no effect on the increases in HR or AUC for the HR response (Fig. 4-1E, F). Figures 4-2 (A-C) are schematic representations of serial coronal sections of the PVN showing the injection sites in 15 saline-treated and 10 E2 (10 pmol)-treated rats.

4.2.2 E2 attenuates the L-glutamate-induced pressor response via ERβ

The ER antagonist, ICI 182,780 (5 pmol), was co-injected with E2 into the PVN 30 minutes prior to L-glutamate and was found to block E2's effects on MAP and AUC for the MAP response (Fig. 4-3A, B). Microinjection of the ERα agonist, PPT (10 and 20 pmol), prior to L-glutamate had no effect on the increases in MAP and the AUC for the MAP response (Fig. 4-3C, D) or on the increases in HR and the AUC for the HR response (data not shown). When the ERα antagonist, MPP (1000 pmol), was co-injected with E2 into the PVN, E2's effect on MAP or the AUC for the MAP response was not affected (Fig. 4-3C, D). Microinjection of the ERβ agonist, DPN (5, 50, and 100 pmol), prior to L-glutamate, attenuated the increases in MAP by up to 57% with 50 pmol DPN and attenuated the increases in AUC for the MAP responses (from 71 ± 34 for saline to -40 ± 27 mm Hg x S, for 50 pmol DPN) (Fig. 4-3E, F). DPN had no effect on the increase in HR (data not shown). When the ERβ antagonist, R,R-THC (5 and 50 pmol), was co-injected with E2, E2's effects on MAP and the AUC for the MAP response were blocked (Fig. 4-3E, F). Microinjections of ICI 182,780 (5 pmol), DPN (100 pmol), PPT (20 pmol), R,R-THC (50 pmol) or MPP (1000 pmol) alone had no effects on baseline MAP and HR.

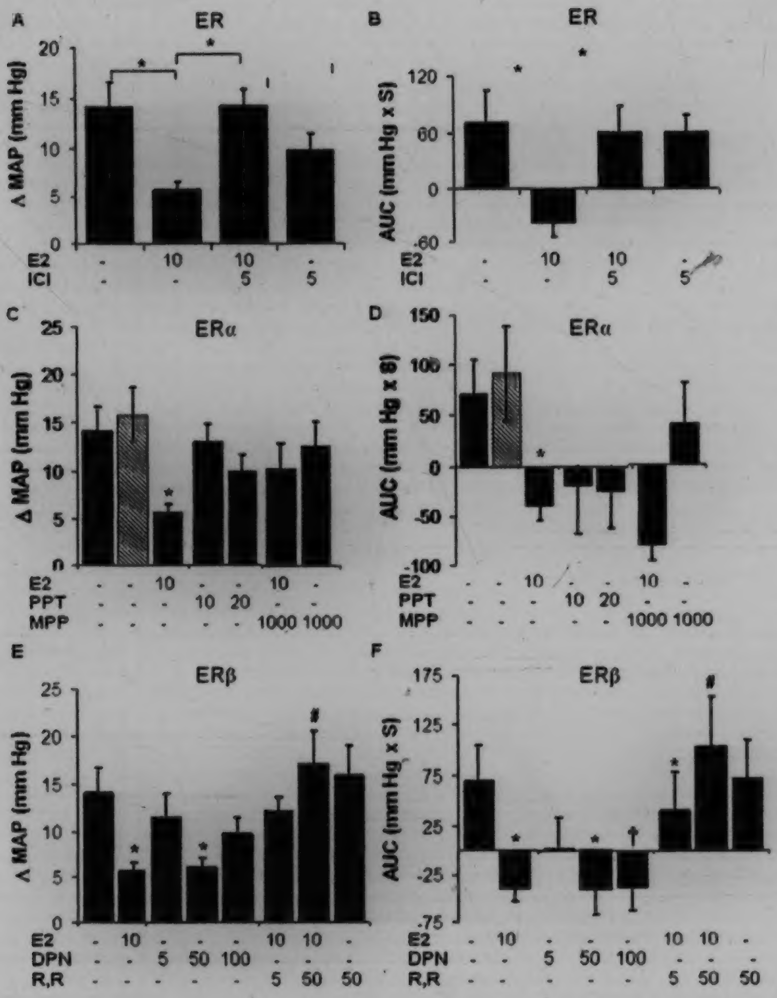

Figure 4-3. Role of ERs in E2's attenuation of the L-glutamate-induced increase in MAP (A) and AUC for the MAP response (B) using the ER antagonist ICI 182,780. Role of ERα on MAP (C) and AUC for the MAP response (D) using the ERα agonist, PPT, and the antagonist, MPP. Role of ERβ on MAP (E) and AUC for the MAP response (F) using the ERβ agonist, DPN, and the antagonist R,R-THC (R,R). Hatched bar represents the water control that contained 10% DMSO used in the MPP experiment. All concentrations are pmol. Data represents mean ± SEM. n≥6. * $p<0.05$

4.2.3 NO mediates E2's attenuation of the L-glutamate-induced pressor response

The non-selective NOS inhibitor, L-NAME (2000 pmol), was co-injected with E2 (10 pmol) into the PVN 30 minutes prior to L-glutamate and was found to block E2's effects on MAP and AUC for the MAP response (Fig. 4-4A, B). The selective nNOS inhibitor, 7-NiNa (0.1 pmol), and the potent eNOS inhibitor, L-NIO (200 pmol), were then each co-injected with E2 (10 pmol); both blocked E2's attenuation of the increases in MAP and the AUC for the MAP response (Fig. 4-4C-F). Microinjection of L-NAME (2000 pmol) alone had no affect on baseline HR, but induced an increase in MAP (13 ± 4 mm Hg) that returned to baseline within 5-10 minutes. Microinjection of 7-NiNa (0.1 pmol) or L-NIO (200 pmol) alone had no effects on baseline MAP or HR.

4.2.4 GABA$_A$ receptors may not be involved in the pathway by which E2 attenuates the L-glutamate-induced pressor response

The GABA$_A$ receptor antagonist, bicuculline (200 pmol), was co-injected with E2 into the PVN 30 minutes prior to L-glutamate. The effect of bicuculline with E2 on the increase in MAP was not significantly different than the effect of E2 alone on the increase in MAP (E2 alone; 6 ± 1 vs. E2 plus bicuculline 10 ± 2 mm Hg; P=0.183). Similarily, the effect of bicuculline with E2 was not significantly different than the effect of E2 alone on the AUC for the MAP response (E2 alone; -38 ± 15 vs. E2 plus bicuculline; 23 ± 21 mm Hg x S; P=0.073) (Fig 4-5A, B). Microinjection of bicuculline (200 pmol) alone induced increases in MAP (11 ± 2 mm Hg) and HR 28 ± 5 beats/min) that returned to baseline within 15-20 minutes.

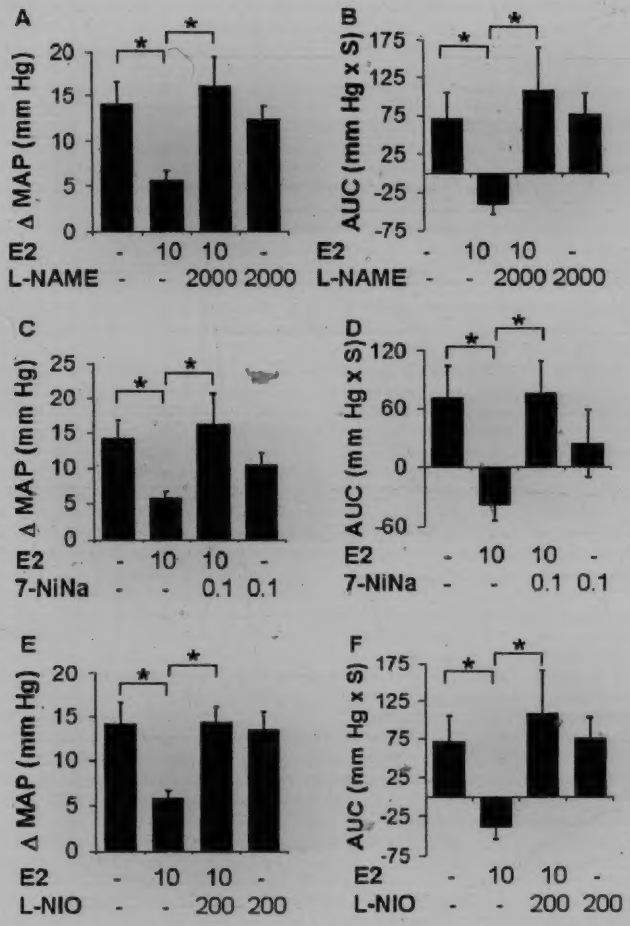

Figure 4-4. Role of NO in E2's attenuation of the L-glutamate-induced increase in MAP (A) and AUC for the MAP response (B) using the non-selective NOS inhibitor L-NAME. Role of nNOS on MAP and AUC for the MAP response (C, D) using the selective nNOS inhibitor 7-NiNa. Role of eNOS on MAP and AUC for the MAP response (E, F) using the potent eNOS inhibitor L-NIO. All concentrations are pmol. Data represented as mean ± SEM. n≥6. * $p < 0.05$.

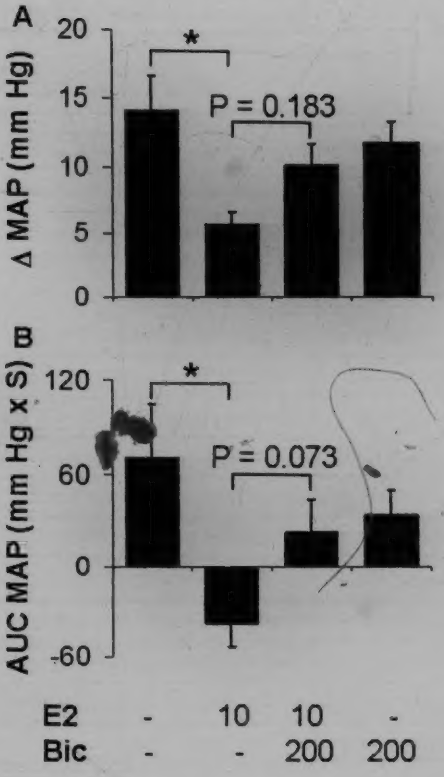

Figure 4-5. Role of GABA in E2's attenuation of the L-glutamate-induced increase in MAP (A) and AUC for the MAP response (B) using the selective $GABA_A$ receptor antagonist, bicuculline (Bic). All concentrations are pmol. Data represented as mean ± SEM. n≥ 6. * $p<0.05$.

4.3 Discussion

In the present study we investigated the effects of E2 in the PVN on resting MAP and HR and on L-glutamate-induced increases in MAP and HR. We show that E2 has no effect on resting MAP or HR, but attenuates the L-glutamate-induced increase in MAP. Using pharmacological agents selective for ERα and ERβ, we also determined that E2's effect is mediated by ERβ. Furthermore, we found that E2 acts on NO produced by nNOS and eNOS in the PVN to attenuate the L-glutamate-induced pressor response. Finally, we show that GABA may not be involved in mediating E2's effect in the PVN.

Our study is the first to investigate the effects of E2 in the PVN on MAP and HR and was carried out in male rats for several reasons. First, we wanted to avoid confounding effects of differing levels of circulating E2 found in cycling females. Second, the expression profiles of ERα and ERβ in the brain, and more specifically in the PVN, are identical in intact male and female rats [217]. Third, previous investigations on the acute effects of E2 on cardiovascular function in other autonomic nuclei were performed in male rats [78, 256]. We found that E2 (0.1 – 10 pmol) has no effect on resting MAP or HR. Similarly, resting BP is unaffected by microinjections of E2 into the nucleus ambiguus [78] and insular cortex [263], and resting HR is unaffected by microinjections into the rostral ventral lateral medulla [78] and insular cortex [263]. In contrast, microinjections of E2 into the PBN and the NTS decrease resting BP and HR [78, 256]. These data suggest that E2's effects on BP and HR in central autonomic nuclei are regionally specific. Because neurons in the PVN of anesthetized animals are quiescent [264, 265] and electrical lesions to the PVN do not affect resting BP and HR in conscious rats [266], we postulate that resting BP and HR are unchanged by E2 in the

PVN because these neurons are less active than neurons of brainstem centers in regulating acute changes in BP during rest.

We tested our hypothesis that E2 modulates BP and HR responses to excitatory stimulation of the PVN in anesthetized rats by investigating the effects of E2 in the PVN on the L-glutamate-induced increases in MAP and HR. Microinjections of L-glutamate (50 nmol, 100 nL) into the PVN increased MAP, HR, and the AUC for the MAP and HR responses. Our results are consistent with previous studies in conscious [267, 268] and anesthetized rats [269] which showed that microinjections of L-glutamate (25 - 100 nmol) into the PVN increase blood pressure by 7-16 mm Hg. We found that E2 (10 pmol) injected into the PVN prior to L-glutamate attenuated the pressor response but did not affect the tachycardia. Similar results were seen in perimenopausal women exposed to mental stress [85] and in OVX rats exposed to restraint stress [86] where E2 supplementation attenuated BP but not HR responses. Because E2's effect on MAP was observed only after the PVN was excited by L-glutamate, our results suggest that E2 acts in the PVN to restore cardiovascular homeostasis in response to perturbations. In support to our findings, others have found that inhibition of PVN neurons with baroreceptor inputs was observed only when these neurons were excited with excitatory amino acids [265]. In order to confirm our hypothesis, however, it will be important to perform these experiments in conscious animals.

We show that E2 injected into the PVN 30 minutes prior to L-glutamate attenuates the pressor response. The rapidity of E2's effect indicates that it is likely mediated by a non-genomic signalling mechanism, further supporting the idea that E2 acts in the PVN to quickly restore cardiovascular homeostasis in response to perturbations. Because rapid non-genomic actions of E2 can be ER-dependent or ER-independent [243], we investigated the role of ERs in E2's effect. We confirmed that E2's effect is mediated by ERs in the PVN, as the non-selective ER antagonist, ICI 182,780, inhibited E2's attenuation of the pressor response. Using the ERβ agonist, DPN, and the ERβ antagonist, R,R-THC, we further determined that activation of ERβ is required for E2 to attenuate the L-glutamate-induced pressor response. We also show that ERα is not involved in E2's effect as the ERα agonist, PPT, and the ERα antagonist, MPP, had no effects on the pressor response. Interestingly, we found that 100 pmol DPN was less effective at attenuating the L-glutamate-induced effects than 50 pmol. This type of biphasic response has been described previously for E2, including the regulation of nNOS activity in cerebellar tissue [270].

We have previously shown that activation of ERβ alters eNOS and nNOS expression in the PVN of rat hypothalamic slice cultures [220]. Activation of ERβ in the PVN also attenuates restraint stress-induced increases in adrenocorticotropic hormone (ACTH), corticosterone plasma levels, and c-FOS expression in the PVN of gonadectomized male rats [90]. These studies show that activation of ERβ in the PVN mediates genomic and non-genomic effects. Our study is the first to demonstrate that activation of ERβ in the PVN plays an important role in the rapid modulation of BP responses to excitatory stimulation. Furthermore, neuronal projections from the PVN to the RVLM, 50% of which express ERβ [271], centrally regulate autonomic function, suggesting that this is a likely pathway through which ERβ activation in the PVN modulates cardiovascular function.

Because NO plays an inhibitory role on sympathetic output in the PVN [150, 175, 260] and we have shown that E2 alters NOS expression in the PVN [220], we hypothesized that E2's attenuation of the L-glutamate-induced pressor response is mediated by NO. Indeed, we found that inhibition of NO with the non-selective NOS inhibitor, L-NAME, blocked E2's effect. We further show th[illegible] NO produced by eNOS and nNOS mediates E2's effect. Similarly, our lab has shown that NO produced by eNOS and nNOS in the PVN mediates adrenomedullin-induced decreases in BP [212]. These current findings further support the hypothesis from our recent *in vitro* study [220], that NO produced by eNOS in blood vessels within the PVN can act on neighbouring neurons to influence autonomic pathways.

GABA in the PVN inhibits sympathetic output [175, 261] and mediates NO-induced inhibition of sympathetic activity [175, 212]. Therefore, we investigated the role of the GABA_A receptor in E2's attenuation of the pressor response. Our results suggest that GABA_A receptors are not involved in mediating E2's effect, as our results showed that the GABA_A receptor antagonist, bicuculline, did not significantly block E2's attenuation of the pressor response. Although our findings suggest that GABA is not involved in E2's attenuation of the pressor response, many other groups have shown that GABA plays an important role in the PVN on regulating cardiovascular function [175, 212, 261]. Therefore, further studies are needed to clearly determine whether GABA in the PVN plays a role in the cardiovascular effects induced by E2 in the PVN.

In this study we have shown that E2 in the PVN of male rats rapidly attenuates the L-glutamate-induced pressor response by activating ERβ to recruit NO. Together with our previous study [86] which demonstrated that E2 acts on NO in the brain to attenuate

BP responses to psychological stress in OVX rats, these findings lead us to hypothesize that E2 mediates its beneficial cardiovascular effects by acting within the PVN to restrain BP responses to stimuli which increase arterial pressure. These results demonstrate that the brain is an important target for E2's effects on cardiovascular function and contribute to our understanding of how E2 provides protection against cardiovascular disease.

Chapter 5

Activation of ERβ increases NO production by increasing phosphorylation levels of nNOS at Ser^{1412} through a Src/PI3/Akt-dependent pathway

5.1 Introduction

Homeostasis is essential for the survival of an organism, ensuring physiological stability amidst variable external surroundings. The homeostatic stress response, orchestrated by the hypothalamus, is modulated by estrogen so that stress responses are smaller in pre-menopausal women compared to age-matched men [272] and in 17β-estradiol (E2)-treated postmenopausal women compared to women not receiving E2 [83, 84]. Similarly, blood pressure responses to restraint stress are attenuated in ovariectomized (OVX) rats receiving E2 compared to OVX rats receiving vehicle [86-88]. Therefore, because elevated blood pressure responses to stress increase the risk of cardiovascular disease [272, 273], E2 is believed to improve cardiovascular health by attenuating the stress response.

A key hypothalamic site for the regulation of the stress response is the paraventricular nucleus (PVN), as it modulates the functions of the sympathetic nervous system and the hypothalamo-pituitary-adrenal axis. Estrogen receptor-β (ERβ) is expressed in pituitary-projecting neurons of the PVN [217] and in 50% of neurons that innervate the brainstem autonomic center, the rostral ventrolateral medulla [271]. Thus, anatomical evidence suggests that the PVN is a target for E2. In OVX rats, E2 attenuates the numbers of activated neurons in the PVN induced by footshock [80] and restraint stress [81]. Furthermore, activation of ERβ in the PVN of male rats using selective ligands attenuates neuronal activation by restraint stress, as determined by c-FOS expression [90]. These studies demonstrate that E2 activates ERβ to modulate the activity of neurons in the PVN involved to restoring homeostatic balance.

We have demonstrated that nitric oxide (NO) mediates the effects of E2 on blood pressure responses to restraint stress and that NO production in the hypothalamus is stimulated by restraint stress in E2-treated, but not vehicle-treated, OVX rats [86]. Recently, we also showed that E2 in the PVN attenuates blood pressure responses to L-glutamate injections into the PVN via ERβ and NO produced by neuronal NO synthase (nNOS) and endothelial NOS (eNOS) [274]. Finally, we demonstrated in hypothalamic slice cultures that E2 regulates nNOS and eNOS expression in the PVN through an ERβ-dependent mechanism [220]. Therefore, our studies show that E2 stimulates the NO system in the hypothalamus through ERβ.

While E2 recruits NO through an ERβ-dependent mechanism in neurons to modulate hypothalamic function, the molecular mechanisms responsible for these effects are unknown. In the present study we used primary hypothalamic neurons to investigate the effects of ERβ activation on nNOS activity and NO production. We tested the hypotheses that ERβ activation increases nNOS phosphorylation at Ser^{1412} and that these effects lead to an increase in NO production. In addition, because little is known about ERβ signalling in neurons, we identified the signal transduction pathway through which activation of ERβ stimulates the NO system in neurons.

5.2 Results

5.2.1 Primary hypothalamic neurons co-express ERβ and nNOS

Using immunocytochemistry we determined that at 8-9 DIV, approximately 90% of the cells in culture were MAP-2-positive and 10% of the cells were GFAP-positive (Fig. 5-1A). The expression of ERβ and nNOS was also studied with immunocytochemistry (Fig. 5-1B) and we found that approximately 90% of neurons in culture were ERβ-positive, a similar proportion to that observed by others [275, 276]. ERβ was localized primarily in the nucleus but cytoplasmic staining was also present in the cell body and neurites. In negative controls in which the primary antibody (ERβ 503 IgY) was omitted from the immunocytochemistry protocol, no detectable signal was observed (data not shown). Others have described the specificity of ERβ 503 IgY using pre-absorption assays [222]. We also determined that 40% of neurons in culture were nNOS-positive. nNOS was localized throughout the cytoplasm of the cell body and neurites. Finally, approximately 40% of neurons were double-labeled for ERβ and nNOS, suggesting that most of the nNOS-positive neurons co-express ERβ.

5.2.2 Activation of ERβ with DPN increases levels of phosphorylated nNOS at Ser^{1412} and NO production

Preliminary time course experiments using E2 (10 nM) were performed to determine the temporal changes in phosphorylation levels of nNOS at Ser^{1412}. We found that E2 stimulated an increase in the levels of Ser^{1412} phosphorylation at 5 minutes; by 15 minutes, levels had returned to baseline (Fig. 5-2A). Based on these results, neurons were treated for 5 minutes in all subsequent experiments. Because we were interested in investigating the effects of ERβ activation on phosphorylation levels of nNOS at Ser^{1412} and on NO production, we next performed dose response experiments using the selective ERβ agonist, DPN (0-1000 nM, 5 min). DPN is 70-fold more selective for ERβ than ERα and exclusively activates ERβ at concentrations used in this study [209, 277]. Activation of ERβ with DPN increased the levels of Ser^{1412} phosphorylation with a maximal increase

A
MAP-2
GFAP
DAPI
Merged
B
nNOS
ERβ
DAPI
Merged

Figure 5-1. (A) Images of primary hypothalamic cultures labeled with the neuronal marker, MAP-2 (green), the astrocyte marker, GFAP (red), and the nuclear marker, DAPI (blue). Merged image shows that cultures are comprised primarily of neurons. (B) Confocal images of hypothalamic neurons labeled with nNOS (green), ERβ (red), and DAPI (blue). In the merged image, arrows indicate neurons that co-express ERβ and nNOS, and arrowheads indicate neurons that express ERβ, but not nNOS. Scale bar in A = 50 μm and in B = 20 μm.

of 57% at 10 nM DPN, compared to vehicle (Fig. 5-2B). While higher concentrations of DPN (100 and 1000 nM) appeared to increase the levels of Ser^{1412} phosphorylation, these effects were not statistically significant. Activation of ERβ with DPN (10 nM and 1000 nM) also stimulated NO production by 38% and 41%, respectively, compared to vehicle (Fig. 5-2C).

5.2.3 PI3K/Akt mediates the increase in levels of phosphorylated nNOS at Ser^{1412} and in NO production following ERβ stimulation

To identify the kinase signalling pathway(s) which is (are) recruited following the activation of ERβ, we first determined whether DPN activated the PI3K and/or the MAPK pathway. We found that DPN (10 nM, 5 min) increased levels of phosphorylated Akt at Ser^{473} by 42% (Fig. 5-3A), but not pERK1/2 (Fig. 5-3B). Using selective inhibitors of the PI3K and MAPK pathways, we next established which kinase pathway mediates the DPN-induced increase in phosphorylation levels of nNOS at Ser^{1412} and in NO production. Neurons were pre-treated with the PI3K inhibitor, LY294002 (10 μM), or the MAPK inhibitor, PD98059 (10 μM), for 30 minutes followed by treatment with vehicle or DPN (10 nM) in the presence of inhibitors for 5 minutes. Neurons pre-treated with vehicle for 30 minutes followed by treatment with vehicle or DPN (10 nM) were used as controls. LY294002 blocked the DPN-induced increase in Ser^{1412} phosphorylation levels while PD98059 did not (Fig. 5-4A). In addition we found that LY294002, but not PD98059, blocked the DPN-induced increase in NO production (Fig. 5-4B). Together these results demonstrate that activation of ERβ stimulates the PI3K/Akt kinase pathway which, in turn, mediates the increases in phosphorylation levels of nNOS at Ser^{1412} and in NO production.

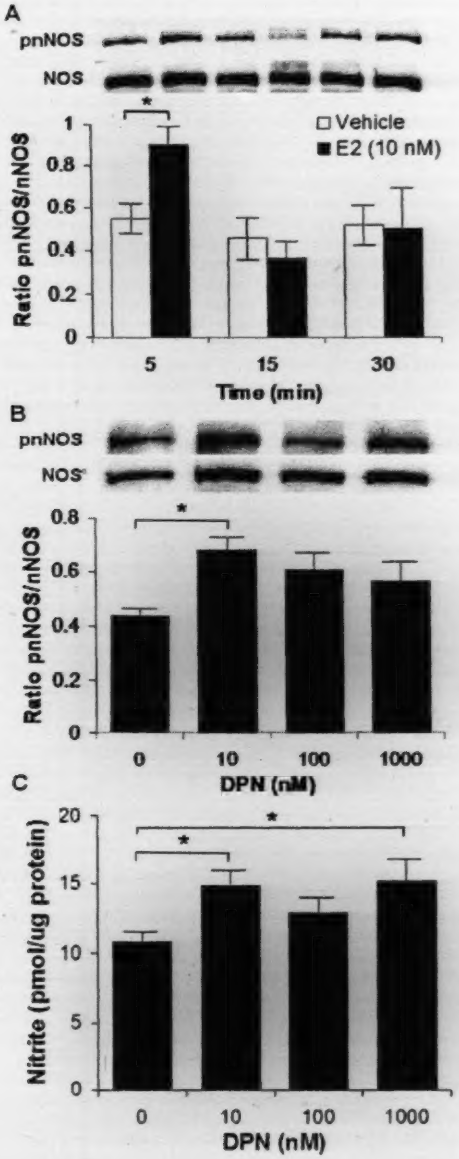

Figure 5-2. Activation of ERs leads to increased phosphorylation levels of nNOS at Ser^{1412} and NO production. (A) Time course effects of 17β-estradiol (10 nM) on phosphorylation levels of nNOS at Ser^{1412} in neurons treated for 5, 15 or 30 min. (B) Dose-response effects of DPN (0- 1000 nM) on phosphorylation levels of nNOS at Ser^{1412} in neurons treated for 5 min. (C) Dose-response effects of DPN (0-1000 nM) on nitrite levels in the culture medium following a 5 min treatment. Data represents mean ± SEM. N = 4 in A, N = 9 in B, and N = 9 in C. In A, * $p<0.05$ vs. time-matched control. In B and C, * $p<0.05$ vs. control.

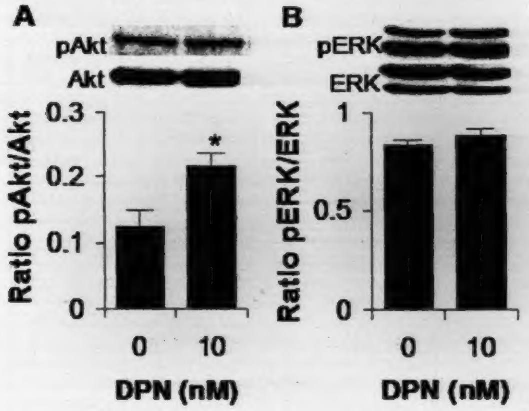

Figure 5-3. Activation of ERβ increases activity of the PI3K/Akt pathway. A and B, Effects of DPN (10 nM, 5 min) on levels of phosphorylated Akt at Ser^{473} and ERK1/2, respectively. Data are represented as mean ± SEM. N = 4. * $p<0.05$ vs. control.

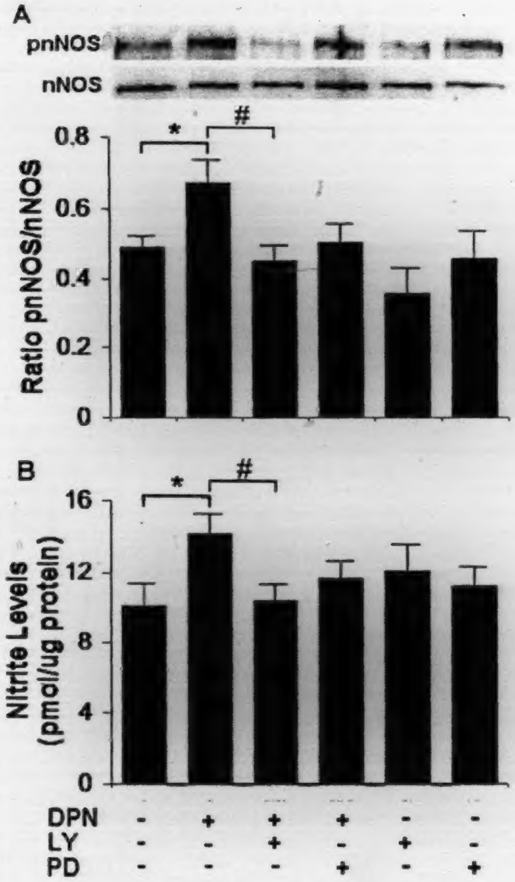

Figure 5-4. Role of the PI3K and MAPK pathways in the effects of ERβ activation on phosphorylation levels of nNOS at Ser^{1412} and NO production. (A) The effects of the PI3K inhibitor, LY294002, and the MAPK inhibitor, PD98095, on the DPN-induced increase in phosphorylation levels of nNOS at Ser^{1412} were determined. Neurons were pre-treated with LY294002 (10 μM) or PD98059 (10 μM) for 30 minutes followed by treatment with vehicle or DPN (10 nM) in the presence of inhibitors for 5 minutes. Other neurons pre-treated with vehicle for 30 min followed by treatment with vehicle or DPN (10 nM) for 5 min were used as controls. (B) Neurons were treated as above and the effects of LY294002 and PD98059 on the DPN-induced increase in nitrite levels were determined. Data are represented as mean ± SEM. N = 5 in A and B. * $p<0.05$ (DPN vs control). # $p<0.05$ (DPN vs. DPN plus LY294002).

5.2.4 Src kinase mediates the increase in levels of phosphorylated nNOS at Ser^{1412} and in NO production following ERβ stimulation

Because the Src kinase pathway is a known mediator of E2's effects and Src is an upstream signalling molecule to PI3K/Akt, we sought to determine whether activation of ERβ by DPN stimulates the Src kinase pathway. DPN (10 nM) treatment for 5 minutes increased the levels of Src phosphorylated at Tyr^{416} by 39% (Fig. 5-5). We used the selective Src inhibitor, PP2, and its inactive analog, PP3, to further establish whether the Src kinase pathway mediates the DPN-induced increase in phosphorylation levels of nNOS at Ser^{1412} and in NO production. Neurons were pre-treated with the inhibitors, PP2 (10 μM) or PP3 (10 μM), for 30 minutes followed by treatment with vehicle or DPN (10 nM) in the presence of the inhibitors for 5 minutes. Neurons pre-treated with vehicle for 30 minutes followed by treatment with vehicle or DPN (10 nM) were used as controls. PP2 blocked the DPN-induced increase in Ser^{1412} phosphorylation levels while the inactive analog did not (Fig. 5-6A). In addition, PP2 but not PP3 blocked the DPN-induced increase in NO production (Fig. 5-6B). Finally, to demonstrate that activation of the Src kinase pathway is acting upstream of the PI3K/Akt pathway to increase levels of phosphorylated nNOS at Ser^{1412}, we investigated the effect of the selective Src inhibitor, PP2, on activation of the PI3K/Akt pathway. We found that blocking Src kinase signalling inhibited the DPN-induced increase in levels of phosphorylated Akt at Ser^{473} (Fig. 5-6C). These results demonstrate that Src kinase mediates ERβ-induced increases in phosphorylation levels of nNOS at Ser^{1412} and in NO production by activating the PI3K/Akt pathway.

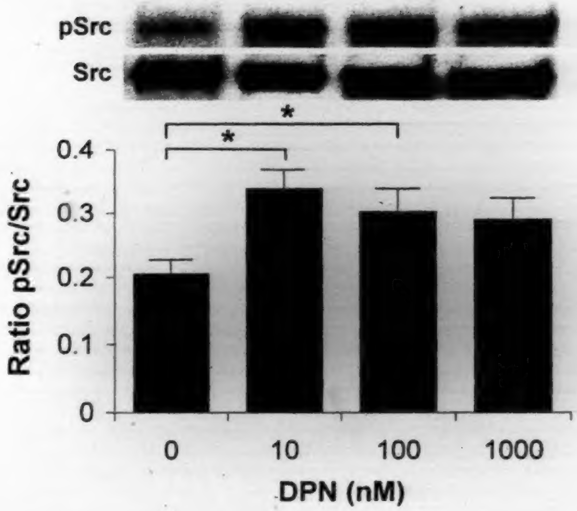

Figure 5-5. Dose-response effects of DPN (0-1000 nM) on phosphorylation levels of phosphorylated Src at Tyr^{416}. Data are represented as mean ± SEM. N = 8. * $p<0.05$ vs. control.

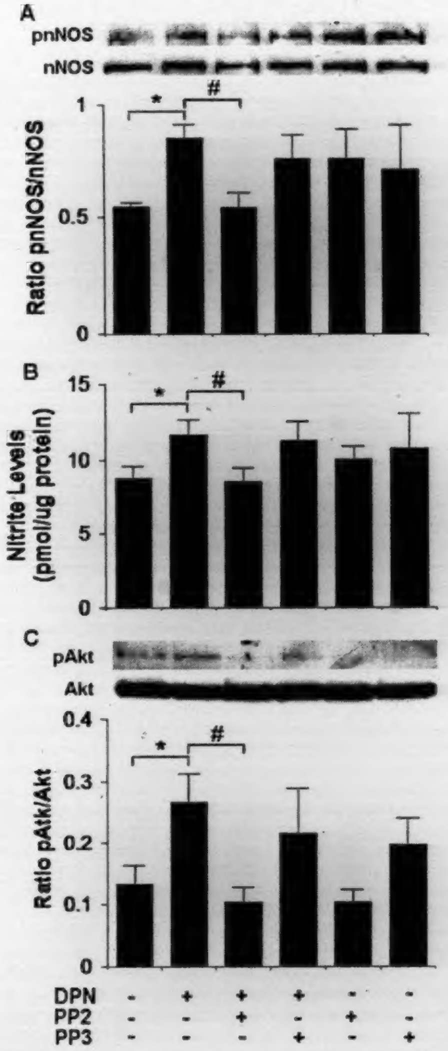

Figure 5-6. Role of the Src kinase pathway in the effects of ERβ activation on phosphorylation levels of nNOS at Ser^{1412}, NO production, and phosphorylation levels of Akt. (A) The effects of the Src inhibitor, PP2, and its inactive analog, PP3, on the DPN-induced increase in phosphorylation levels of nNOS at Ser^{1412} were determined. Neurons were pre-treated with PP2 (10 µM) or PP3 (10 µM) for 30 minutes followed by treatment with vehicle or DPN (10 nM) in the presence of inhibitors for 5 minutes. Other neurons pre-treated with vehicle for 30 min followed by treatment with vehicle or DPN (10 nM) for 5 min were used as controls. (B) and (C) Neurons were treated as above and the effects of PP2 and PP3 on the DPN-induced increase in nitrite levels and levels of phosphorylated Akt at Ser^{473}, respectively, were determined. Data are represented as mean ± SEM. N = 4 in A and C, and N = 7 in B. * $p<0.05$ (DPN vs control). # $p<0.05$ (DPN vs. DPN plus PP2).

5.3 Discussion

We show here for the first time that activation of ERβ in rat primary hypothalamic neurons increases NO production by regulating nNOS activity through its phosphorylation. First, we show that activation of ERβ with the selective agonist, DPN, rapidly increases phosphorylation levels of nNOS at Ser^{1412} and increases NO production. Second, we demonstrate that activation of ERβ stimulates the Src and PI3K/Akt pathways. Finally, we determine that the increases in phosphorylation levels of nNOS at Ser^{1412} and in NO production induced by ERβ activation are mediated by the Src and PI3 kinase pathways. Thus, our results, schematically represented in Figure 5-7, demonstrate that, activation of ERβ stimulates the NO system in neurons through a PI3K/Akt-dependent mechanism mediated by Src kinase.

Several studies provide indirect evidence that E2 increases NO production from neurons. We and others have shown that E2 increases NO production from neuroblastoma cells [121, 200] and from median eminence explants from male [201] and OVX rats [199]. In addition, preoptic [198] and median eminence explants [199] dissected from proestrus female rats (when circulating E2 is high) produce more NO than explants from diestrus female rats (when E2 is low). Neither the cell type(s) responsible for NO production nor the mechanisms were determined in these studies, and it is possible that non-neuronal cells, such as endothelial and glial cells, present in these models contributed substantially to the increase in NO induced by E2. Therefore, in order to investigate whether activation of ERβ increases NO production from neurons and to minimize the presence of other cell types, we used primary hypothalamic neurons in the present study. We now provide direct evidence that activation of ERβ increases NO production from neurons, although we cannot rule out a small contribution from non-neuronal cells.

Interestingly, the E2-induced increases in NO production described in earlier studies were not accompanied by an increase in nNOS protein expression, but instead, coincided with increases in NOS activity [121, 198], suggesting that E2 increases NO production via post-translational modifications of nNOS. Because biochemical evidence suggests that phosphorylation of the nNOS protein at Ser^{1412} increases nNOS activity [278], we tested the hypothesis that ERβ activation modulates nNOS phosphorylation levels at this site in primary hypothalamic neurons. Indeed, we found that ERβ activation increases the levels of phosphorylated nNOS at Ser^{1412}. During the course of our study, Rameau et al. demonstrated in cortical neurons that glutamate treatment increases phosphorylation levels of nNOS at Ser^{1412} and that these effects, in turn, lead to increased nNOS activity [159]. The time course of the increase in phosphorylation levels of nNOS at Ser^{1412} which we observed following E2 treatment is similar to that of Rameau et al. following glutamate treatment, such that phosphorylation levels of Ser^{1412} reach a maximal increase 5 minutes after treatment and decline to basal levels by 15 minutes.

Because activation of ERβ increases nNOS activity by enhancing phosphorylation levels of nNOS at Ser^{1412}, we speculated that the MAP kinase (MAPK) or the PI3/Akt serine/threonine kinase pathway mediates this effect based on the findings that, in neurons, E2 rapidly activates both pathways [54, 55, 58, 141]. We found that, in primary hypothalamic neurons, activation of ERβ rapidly activates the PI3K/Akt

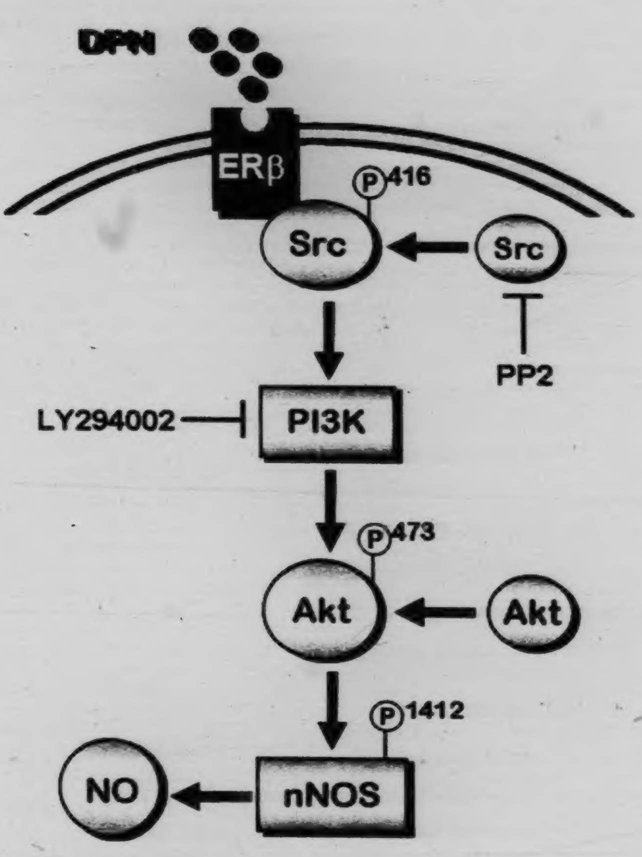

Figure 5-7. Model of the signal transduction pathway initiated by ERβ activation and leading to increased NO production in hypothalamic neurons. Stimulation of ERβ by the selective agonist, DPN, rapidly activates the Src kinase pathway by increasing levels of phosphorylated Src at Tyr^{416}. Stimulated Src kinase then activates the PI3K/Akt pathway by increasing the levels of phosphorylated Akt at Ser^{473}. Activated Akt phosphorylates nNOS at Ser^{1412}, resulting in an increase in nNOS activity which leads to the subsequence synthesis and release of NO from hypothalamic neurons. The increase in NO production induced by the activation of ERβ is inhibited by the Src kinase inhibitor, PP2, and the PI3 kinase inhibitor, LY294002, shown at their sites of action.

pathway, but not the MAPK pathway, and that the PI3K/Akt pathway mediates the ERβ-induced increases in phosphorylation levels of nNOS at Ser^{1412} and in NO production. Our findings are supported by the presence of a putative Akt phosphorylation motif at Ser^{1412} [278, 279] on the nNOS protein and by the demonstration that Akt increases phosphorylation levels of nNOS at Ser^{1412} in cortical neurons [159].

Because ERs do not contain an intrinsic kinase domain they must physically interact with other signalling molecules that activate downstream cascades to mediate non-genomic effects [280, 281]. Steroid receptor co-activator (Src) is a tyrosine kinase recruited by E2 to initiate rapid non-genomic effects in a variety of cell types including neurons, endothelial cells, and cancer cells [58, 123, 280, 282]. While Src mediates E2-induced increases in MAPK activity in cortical [55], cerebellar [283], and hippocampal neurons [58], and E2 increases Src kinase activity via ERβ in platelets [284], it was not known whether Src acts as a downstream effector of ERβ in neurons. We show here that activation of ERβ in neurons increases Src kinase activity and that, following ERβ activation, Src kinase mediates PI3K/Akt-dependent increases in phosphorylation levels of nNOS at Ser^{1412} and in NO production, as the Src kinase inhibitor PP2 blocked the effects of ERβ activation.

The rapid non-genomic effects of ERβ activation demonstrated in this study are presumably carried out by ERβ located in the cytoplasm or at the membrane. While we found that ERβ is localized predominantly in the nucleus of hypothalamic neurons, cytoplasmic staining in the soma and neurites was also observed. ERβ immunoreactivity has also been described in the cytoplasm of hippocampal [101, 126, 285] and cortical neurons [101]. In addition, others have demonstrated that ERβ is localized at the membrane in hippocampal neurons [126], NG108-15 neuroblastoma cells [286], endothelial cells [125], and platelets [284].

The suggestion that classical ERs are located in the plasma membrane has been questioned as ERs lack a trans-membrane domain. However, ERβ has been shown to be localized to the plasma membrane by palmitoylation in transfected Chinese hamster ovary cells [129]. In human colon cancer (DLD-1) cells, endogenous ERβ associates with the plasma membrane-specific protein, caveolin-1, when palmitoylated [130]. In addition, ERβ has been shown to mediate rapid E2-induced eNOS activation in caveolae, the membrane domain that expresses caveolin-1, of endothelial cells [127]. Although caveolae have been identified in hippocampal [287] and dorsal root ganglion neurons [288], ERβ has yet to be identified in caveolae of neurons. Further studies are needed to determine whether palmitoylation of ERβ or association of ERβ with caveolae is important in neurons.

We demonstrate here that activation of ERβ increases nNOS phosphorylation levels and NO production through a Src/PI3K/Akt-dependent pathway in primary hypothalamic neurons. While this is the first study to examine the effects of ERβ on nNOS phosphorylation in neurons, similar findings have been observed in endothelial cells whereby E2 rapidly increases eNOS activity and NO production through a PI3K/Akt-dependent pathway mediated by Src [123]. The effects of E2 in endothelial cells were further determined to be mediated by the rapid formation of a signalling complex containing ER, Src, and p85α, the regulatory subunit of PI3K, [123]. Although the study by Haynes et al. was focused on the activation of eNOS, the similarities between this study and ours raise the intriguing possibility that nNOS is activated in neurons by an ERβ/Src/PI3K signalling complex. It was recently demonstrated in primary cortical neurons that ER(s) directly interact(s) with p85α [143]. However, the identity of the ER involved is unknown as the anti-ER antibody used in this study recognizes both ERα and ERβ [143]. Furthermore, while Simmoncini *et al.* reported that ERβ does not interact with p85α in endothelial cells [188], Moro *et al.* determined that ERβ does interact with p85α in enucleated platelets [284]. Future studies are needed to characterize the signalling complex responsible for ERβ's activation of nNOS and to better understand the mechanisms through which E2 stimulates NO production in neurons.

In conclusion, our findings that activation of ERβ increases levels of phosphorylated nNOS and NO production through a Src/PI3K/Akt-dependent pathway (Fig. 5-7) provide insight into the signalling mechanism through which E2 modulates hypothalamic function. Our current findings, together with our previous demonstration that NO in the hypothalamus mediates E2's attenuation of blood pressure responses to restraint stress in OVX rats [86], lead us to hypothesize that activation of ERβ increases NO production in hypothalamic neurons and that these effects attenuate sympathetic output and blood pressure responses to stress. In addition to mediating the effects of E2 on sympathetic output [86, 274], NO has also been shown to mediate the effects of E2 on neuroprotection [200, 205], dendritic branching [206] and prolactin release [289]. Therefore, because ERβ is the predominant ER in the brain [100], our results raise the intriguing possibility that increased NO production by ERβ activation constitutes a major signalling pathway through which E2 modulates neuronal activity.

Chapter 6

Conclusions and Perspectives

6.1 Synopsis

Estrogen plays an important role in many physiological systems including the reproductive, skeletal, cardiovascular, and central nervous system. Our lab previously demonstrated that 17β-estradiol (E2) attenuates blood pressure responses to restraint stress by stimulating NO production in the brain. However, the specific site in the brain where E2 stimulates the NO system was unknown. By using *in vitro* and *in vivo* models, we provide evidence that the PVN of the hypothalamus is a key site where E2, through ERβ activation, modulates the NO system to regulate autonomic output. We further describe the signalling mechanisms through which activation of ERβ stimulates NO in hypothalamic neurons.

Using hypothalamic slice cultures we showed that E2 increases eNOS protein expression in the PVN and decreases the numbers of nNOS-positive neurons in the PVN through a slow, genomic effect. Because the changes in eNOS and nNOS expression were specific to the PVN, our findings demonstrate that the PVN is a specific target for E2's effects on the NO system. Finally, by using a variety of selective ER agonists and antagonists we demonstrated that ERβ, but not ERα, is responsible for E2's effect on NOS expression in the PVN.

In order to determine the physiologial effects of E2 in the PVN and the potential role of NO in these effests, we performed microinjections of E2 into the PVN of anesthetized male rats. We showed that E2 has no effect on resting MAP or HR, but attenuates the L-glutamate-induced increase in MAP. Using pharmacological agents selective for ERα and ERβ, we also determined that E2's effect is mediated by ERβ. Furthermore, we found that E2 acts on NO produced by nNOS and eNOS in the PVN to attenuate the L-glutamate-induced pressor response. Finally, we show that GABA partially mediates E2's effect in the PVN.

By using primary hypothalamic neurons we were able to identify the signal transduction pathway initiated by ERβ activation that stimulates the NO system. We showed that activation of ERβ in rat primary hypothalamic neurons increases NO production by increasing nNOS activity through its phosphorylation. We also determined that the increases in phosphorylation levels of nNOS and in NO production induced by ERβ activation are mediated by a Src/PI3K/Akt pathway.

6.2 Perspectives

Hormone replacement therapy (HRT) has been used to treat menopausal symptoms since the early 1940's and was believed to protect postmenopausal women against cardiovascular disease [252-254]. However, clinical trials investigating the long-term benefits and risks of HRT were not initiated until 50 years later. The Women's Health Initiative (WHI) was one of the largest clinical trials aimed at investigating the effects of long-term HRT on heart disease, fractures, and breast and colorectal cancer in postmenopausal women [223]. From 1993 to 1998 over 16,000 post-menopausal women aged 50-79 were recruited. Participants were considered predominantly healthy, as only 7.7% reported having prior cardiovascular disease [223]. To the surprise of many, the WHI study ended abruptly in 2002 after an average of 5.2 years of follow-up. Data indicated that HRT increased the risks of breast cancer, coronary heart disease, stroke, and pulmonary embolism [223]. Although data from the WHI also indicated that HRT decreased the risks of hip fractures and colorectal cancer, these benefits did not outweigh the increased risk of conditions mentioned above.

The findings of the WHI study sparked a massive debate in the scientific community over the use of HRT and left postmenopausal women confused about the protective benefits of HRT. The WHI study has received a variety of criticisms, ranging from the design of the WHI study to the validity of its findings based on improper statistical analysis [290, 291]. Some suggest that lower doses of hormone therapy, non-oral routes of administration, and initiation of HRT closer to the onset of menopause may minimize some of the risks observed in the WHI trial [290, 292]. Nonetheless, there is a consensus that the findings from this study emphasize the importance of understanding the effects of estrogen and the mechanisms though which estrogen acts on human physiology. Furthermore, because women now live up to 1/3 of their lives in a postmenopausal state and it is estimated by the year 2030 that the number of postmenopausal women worldwide will reach 1.2 billion [293], there is an overwhelming need to better understand how estrogen affects human health and the physiological consequences of estrogen deficiency.

Anatomical studies have revealed that estrogen receptors are abundant throughout the CNS [100], which is now appreciated as a key site of estrogen action. Although the effects of 17β-estradiol (E2) on development, memory, and neuroprotection have received a lot of attention, the effects of E2 in autonomic centers of the CNS involved in regulating cardiovascular function are less well understood. Cardiovascular disease is the leading cause of death of postmenopausal women [18]. Elevated blood pressure responses to stress increase the risk of cardiovascular disease [272, 273] and E2 is suggested to improve cardiovascular health by attenuating stress responses. Because homeostatic stress responses are regulated by the hypothalamus, it is of particular importance to understand the molecular mechanisms of E2 action and its effects in the hypothalamus on blood pressure responses to stress.

Our laboratory has previously demonstrated that E2 attenuates blood pressure responses to restraint stress in OVX rats and that these effects are mediated by NO in the brain [86]. However, the specific site in the brain where E2 stimulates NO to modulate blood pressure responses was unknown. Because restraint stress increases NO production in the hypothalamus of OVX-E rats [86] we were interested in investigating the effects of E2 on the NO system in a cardiovascular center of the hypothalamus, the paraventricuar nucleus (PVN). Our findings demonstrate that E2 alters the expression of eNOS and nNOS through an ERβ-dependent mechanism in the PVN of hypothalamic slice cultures. Because the changes in NOS expression were observed following a minimum of 8 or 24 hours of E2 treatment, these findings suggest that E2 acts through a genomic mechanism to induce changes in NOS gene transcription. E2 likely increases eNOS expression in the PVN through ERβ/ERE-dependent signalling, as the promoter region of eNOS contains an ERE. On the other hand, the promoter region of nNOS does not contain an ERE, but instead contains binding sites for SP-1 and AP-1. Because ERβ represses AP-1 transcription [115], the mechanism through which E2 decreases nNOS expression in the PVN is likely through ERβ/AP-1-dependent signalling.

We were further interested in investigating the cardiovascular effects of E2 in the PVN. We provide for the first time direct evidence that E2 acts in the PVN through ERβ and NO to regulate blood pressure responses to stimulation. Because other autonomic nuclei in the CNS involved in regulating cardiovascular function, such as the NTS and RVLM, also contain NO-producing neurons [150] and estrogen receptors [100, 271], it is possible that E2 also stimulates NO production in these nuclei to regulate blood pressure responses. By demonstrating that NO produced by nNOS and eNOS in the PVN mediates the effects of E2 on blood responses to stimulation, our findings suggest that NO produced by eNOS in blood vessels signals to adjacent neurons and plays an important role in the regulation of neuronal function by NO. Our suggestion has since been confirmed by a study that showed NO produced by eNOS signals to axons of neurons to affect their activity in optic nerve preparations [294].

Because the first estrogen receptor, ERα, was discovered only 20 years ago, followed 10 years later by the discovery of ERβ, our understanding of E2 signalling is still incomplete. In addition to E2 carrying out its effects through slow genomic signaling, rapid effects are carried out through non-genomic signaling pathways, the latter of which have received a great deal of attention in the past 8 years. Most research on non-genomic signalling of E2 is carried out in endothelial cells, although non-genomic signalling in neurons is also demonstrated. The role of each receptor subtype in non-genomic estrogen signalling is rarely identified and those studies that do, often single out ERα. ERβ is often overlooked as a player in E2 signalling, even though it has been demonstrated as the predominant receptor in endothelial cells [191, 192] and in the brain [100].

Clearly identifying the role of each receptor subtype has been difficult in the past, as selective ligands for each receptor subtype were not available. Without the use of selective ligands researchers relied upon transgenic mice to delineate the roles of ERα and ERβ. However, ER, ERα and ERβ knockout mice are expensive and have compromised fertility. Fortunately, within the past five years several highly selective agonists and antagonists for ERα and ERβ have been developed. With these ligands researchers can now effectively delineate the specific roles of ERα and ERβ in estrogen signalling. Complicating matters even more was the recent identification of the putative estrogen G-protein coupled receptor, GPR30. Although GPR30 has not been clearly established as a functional estrogen receptor *in vivo,* the findings thus far are promising [99, 295]. Future studies are sure to provide insight into the functional role of GPR30 in E2 signalling.

Here we provide one of the first accounts that ERβ plays an important role in rapid non-genomic E2 signalling in the brain. Specifically, by using selective ER ligands we demonstrated that E2 stimulates the NO system exclusively through ERβ in the PVN to modulate autonomic function. Because the role of ERβ in non-genomic signalling by estrogen is relatively unknown, the molecular mechanisms of non-genomic E2 signalling through ERβ have not been identified. Our study here is the first to identify the signal transduction pathway initiated following activation of ERβ in hypothalamic neurons that leads to the stimulation of the NO system. Because ERβ is the predominant ER in the brain and NO mediates many of E2's effects here, the signal transduction pathway we describe may constitute a major signalling pathway through which E2 modulates neuronal activity.

Together the findings from our three studies demonstrate that E2 modulates the NO system through genomic and non-genomic signalling mechanisms. Specifically we demonstrate: 1) that E2 in the PVN of hypothalamic slice cultures modulates NOS expression through genomic-signalling mechanism, 2) that E2 in the PVN, through ERβ and NO, rapidly attenuates blood pressure responses to L-glutamate stimulation, suggesting that E2 acts through a rapid non-genomic signalling pathway, and finally 3) that ERβ through rapid non-genomic actions on nNOS phosphorylation stimulates NO production from hypothalamic neurons.

By demonstrating that E2 modulates the NO system both at the genomic and at the post-translational level, these findings lead us to speculate that E2 simultaneously activates both pathways. By doing so, E2 modulates the activity of NOS and the production of NO to elicit very rapid responses, and at the same time, initiates changes in gene transcription of NOS to carry out long-term responses. Although we did not demonstrate that E2 modulates both post-translational modifications and transcriptional changes in NOS in the same system, one might speculate this to be the case, as non-genomic and genomic actions of E2 have been shown to converge [111]. Simultaneous initiation of non-genomic and genomic actions on the NO system by E2 would provide a more complex level of control, compared to two pathways functioning independent of each other. Future studies are needed to unravel the complex interactions between estrogen and the NO system in the brain.

The findings described herein demonstrate that E2, via ERβ and NO, attenuates blood pressure responses to stimulation. Because elevated blood pressure responses to stimuli such as stress increase the risk of cardiovascular disease [272, 273], our findings provide insight into the mechanisms through which E2 may protect women from stress-induced increases in blood pressure, thus leading to improved cardiovascular health.

Attenuated stress responses not only benefit cardiovascular health, but other physiological systems as well. Stress negatively affects the reproductive systems of both females and males. In females many aspects of the hypothalamic-pituitary-gonadal axis are inhibited by stress-induced activation of the hypothalamic-pituitary-adrenal axis [296]. Specifically, GnRH and LH secretion are inhibited, resulting in inhibition of ovulation [296, 297]. The reproductive behavior of lordosis in female rats is also attenuated following stress [297]. In male rats, stress reduces spermatogenesis and inhibits sexual drive [298]. In humans, psychological and psychosocial stress inhibits reproductive function leading to infertility [299, 300]. Therefore, attenuated responses to stress, through the mechanisms described here, may perhaps benefit reproductive health and improve fertility in humans.

6.3 Future Directions

6.3.1 Determine the role of Ca^{++} in ERβ signalling

E2 has been shown to increase intracellular calcium (Ca^{++}) in several cell types including neurons [58, 301]. Ca^{++} appears to play an important role in a variety of E2's non-genomic effects in neurons. We have previously shown that E2-induced increase in NO production from neuroblastoma cells is dependent on both the influx of extracellular Ca^{++} through L-type channels, as well as the release of Ca^{++} from intracellular stores [121]. Others have described that Ca^{++} mediates the E2-induced activation of the PI3K/Akt pathway [141] and the Src kinase pathway [58] in neurons. The role of Ca^{++} in mediating the effects of ERβ activation is unknown. Given that we described in Chapter 5 that activation of ERβ increases NO production and activates the Src and PI3/Akt kinase pathways, it is likely that Ca^{++} is involved in mediating these effects. First, by using a membrane permeable Ca^{++} sensitive dye such as fura-2 or Calcium Orange, the effect of ERβ activation in neurons on Ca^{++} dynamics can be visualized and measured in order to determine if Ca^{++} is a downstream signalling molecule to ERβ in neurons. Further experiments using nifedipine, an inhibitor of L-type Ca^{++} channels, or BAPTA/AM, a chelator of intracellular Ca^{++}, would determine if Ca^{++} from either extracellular sources or internal stores mediates the effects of ERβ activation on NO production and activation of the Src and PI3/Akt pathways.

6.3.2 Characterize the ERβ signalling complex that stimulates nNOS to increase NO production in neurons

We described in Chapter 5 that activation of ERβ rapidly activates nNOS and stimulates NO production in hypothalamic neurons through a Src/PI3K/Akt-dependent pathway. It has been demonstrated in endothelial cells that a signalling complex containing ER, Src, and P85α, the regulatory subunit of PI3K, is rapidly formed following E2 treatment and leads to the activation of eNOS and an increase in NO production [123]. We hypothesize that the rapid effects following activation of ERβ involve the formation of a similar signalling complex in neurons. Using immunoprecipitation techniques we can identify members of the ERβ signalling complex, such as Src and the P85α the regulatory subunit of PI3K.

6.3.3 Role of MNAR in ERβ signalling in neurons

A novel scaffolding protein, named modulator of non-genomic activity of ER (MNAR), has recently been shown to regulate the interaction between Src and ERα [302] and to mediate the activation of the PI3K [303] and MAPK [302] pathways by E2 in cancer cells. MNAR is co-expressed with ERα in neurons of various brain regions including, the hypothalamus, cortex and hippocampus [304]. We hypothesize that MNAR is involved in non-genomic signalling by ERβ in neurons. Using commercially available antibodies against MNAR we can determine by immunofluorescence if MNAR is co-expressed in ERβ-immunoreactive neurons and by using immunoprecipitation techniques we can further determine if ERβ physically interacts with MNAR. In addition, by inhibiting the activity of MNAR we can determine its role in the effects of ERβ-dependent non-genomic signalling in neurons described in Chapter 5. Because selective inhibitors of MNAR do not exist at this time, we can use siRNA to block expression and activity of MNAR in order to identify its role in ERβ signalling in neurons.

6.3.4 Characterize the interaction between ERβ and the plasma membrane in neurons

Rapid non-genomic actions of E2 are likely carried out by membrane ERs. Although ERs lack a trans-membrane domain, ERβ has been demonstrated to localize to the plasma membrane through palmitoylation [129, 130] and through the association of the plasma membrane protein, caveolin-1[127, 130]. ERβ localization at the plasma membrane has been described in neurons of the hippocampus [126] and in NG108-15 neuroblastoma cells [286]. Using primary neurons such as hypothalamic neurons described in Chapter 5 that abundantly express ERβ, we can first demonstrate whether ERβ is expressed at the plasma membrane by performing electron microscopy. Because of the greater resolution obtained using electron microscopy, it is a much more sensitive detection method compared to light microscopy and allows for discrete immunoreactivity to be detected. Another method that can be used to identify ERβ expression at the plasma membrane is to separate plasma membrane fractions from whole cell lysates and then probe for ERβ using western blotting. Immunoprecipitation studies can be used to identify if ERβ in plasma membrane fractions binds to caveolin-1. Because palmitoylation is required for ERβ to localize to the plasma membrane in cancer cells, we can investigate if the same is true in neurons. To determine if ERβ in neurons is palmitoylated, neurons are treated with [^{3}H]-palmitate, ERβ is immunoprecipitated and the amount of [^{3}H]-palmitate bound to ERβ is counted. Furthermore, to determine if palmitoylation of ERβ is required for non-genomic signalling in neurons, we can inhibit palymitoylation using 2-bromopalmitate, the inhibitor of palmitoyl acyl transferase, the enzyme responsible for palmitoylating proteins, and investigate its effects on the ERβ induced activation of Src, PI3K, nNOS and NO production in neurons.

6.3.5 Role of GPR30 in NO signalling

Recently the G-protein coupled receptor, GPR30, has been identified as a novel membrane ER [98]. Although some still speculate whether GPR30 is a bona fide ER *in vivo*, earlier this year GPR30 was found to be present throughout the rat CNS and in primary hypothalamic neurons [99]. In addition, GPR30 has been shown to mediate rapid effects of E2 on intracellular Ca^{++} concentrations [99, 107, 131] and cAMP activity [97, 107, 132]. Although the role of GPR30 in E2's non-genomic actions in neurons is relatively unknown, one might suggest that it is involved in a wide variety of neuronal pathways. By using the commercially available selective ligand for GPR30, G-1, we can investigate the effects of GPR30 activation on the NO system in hypothalamic neurons.

6.3.6 Determine if activation of ERβ in the PVN attenuates blood pressure responses to restraint stress in conscious animals

We have previously shown that NO in the brain mediates E2's attenuation of blood pressure responses to restraint stress in OVX rats [86]. In addition, we describe in Chapter 4, that E2 acts in the PVN through ERβ and NO to attenuate blood pressure responses to L-glutamate stimulation. From these findings we hypothesized that E2 acts in the PVN to restore cardiovascular homeostasis in response to perturbations. In order to confirm this hypothesis, experiments that investigate the effects of E2 in the PVN on blood pressure responses to restraint stress in conscious male, OVX-V and OVX-E can be performed. Like those experiments in Chapter 4, we can use selective ligands for ERs and NOS to determine the role of ERβ and NO in E2's effects in the PVN on blood pressure responses to restraint stress. In addition we can use the selective ERβ agonist, DPN, which was previously unavailable, to determine the role of ERβ.

6.4 Concluding Remarks

The studies described herein combine *in vivo* and *in vitro* approaches to demonstrate that E2 acts through ERβ to regulate autonomic output from the PVN of the hypothalamus and establish the molecular mechanisms through which activation of ERβ carries out its effects. As the field of estrogen research progresses we must continue to characterize the effects of estrogen on human physiology, the signal transduction pathways that carry out E2's effects, and the specific roles of each receptor subtype. Future studies investigating: 1) the physiological role of ERβ activation in homeostatic responses such as stress, and 2) the signalling machinery involved in ERβ's rapid effects in neurons will strengthen our understanding of ERβ's role in estrogen signalling. By clearly understanding how E2 signals in various physiological systems we will be better equipped to developed specific hormone therapies without deleterious side effects that will improve the health of men and women.

Chapter 7

References

1. Allen, E. and E.A. Doisy, *Landmark article Sept 8, 1923. An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. By Edgar Allen and Edward A. Doisy.* Jama, 1983. **250**(19): p. 2681-3.
2. Emmen, J.M. and K.S. Korach, *Estrogen receptor knockout mice: phenotypes in the female reproductive tract.* Gynecol Endocrinol, 2003. **17**(2): p. 169-76.
3. Messinis, I.E., *From menarche to regular menstruation: endocrinological background.* Ann N Y Acad Sci, 2006. **1092**: p. 49-56.
4. Kalra, S.P., *Observations on facilitation of the preovulatory rise of LH by estrogen.* Endocrinology, 1975. **96**(1): p. 23-8.
5. Herbison, A.E., *Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons.* Endocr Rev, 1998. **19**(3): p. 302-30.
6. Smith, M.J. and L. Jennes, *Neural signals that regulate GnRH neurones directly during the oestrous cycle.* Reproduction, 2001. **122**(1): p. 1-10.
7. Girling, J.E. and P.A. Rogers, *Recent advances in endometrial angiogenesis research.* Angiogenesis, 2005. **8**(2): p. 89-99.
8. Prins, G.S., et al., *The role of estrogens in normal and abnormal development of the prostate gland.* Ann N Y Acad Sci, 2006. **1089**: p. 1-13.
9. Couse, J.F. and K.S. Korach, *Estrogen receptor null mice: what have we learned and where will they lead us?* Endocr Rev, 1999. **20**(3): p. 358-417.
10. O'Donnell, L., et al., *Estrogen and spermatogenesis.* Endocr Rev, 2001. **22**(3): p. 289-318.
11. Turner, R.T., B.L. Riggs, and T.C. Spelsberg, *Skeletal effects of estrogen.* Endocr Rev, 1994. **15**(3): p. 275-300.
12. Deroo, B.J. and K.S. Korach, *Estrogen receptors and human disease.* J Clin Invest, 2006. **116**(3): p. 561-70.
13. Sagraves, R., *Estrogen therapy for postmenopausal symptoms and prevention of osteoporosis.* J Clin Pharmacol, 1995. **35**(9 Suppl): p. 2S-10S.
14. Ettinger, B., H.K. Genant, and C.E. Cann, *Long-term estrogen replacement therapy prevents bone loss and fractures.* Ann Intern Med, 1985. **102**(3): p. 319-24.
15. Jeong, K.S., et al., *Measurement of estrogen effect on bone turnover by 2H2O labeling.* Calcif Tissue Int, 2005. **76**(5): p. 365-70.
16. Wronski, T.J., et al., *Estrogen treatment prevents osteopenia and depresses bone turnover in ovariectomized rats.* Endocrinology, 1988. **123**(2): p. 681-6.
17. Turgeon, J.L., et al., *Hormone therapy: physiological complexity belies therapeutic simplicity.* Science, 2004. **304**(5675): p. 1269-73.
18. Rosano, G.M., C. Vitale, and M. Fini, *Hormone replacement therapy and cardioprotection: what is good and what is bad for the cardiovascular system?* Ann N Y Acad Sci, 2006. **1092**: p. 341-8.
19. Guo, X., et al., *Estrogen induces vascular wall dilation: mediation through kinase signaling to nitric oxide and estrogen receptors alpha and beta.* J Biol Chem, 2005. **280**(20): p. 19704-10.
20. Nechmad, A., et al., *Estrogen induces nitric oxide-mediated vasodilation of human mammary arteries in vitro.* Nitric Oxide, 1998. **2**(6): p. 460-6.

21. Kayisli, U.A., et al., *Regulation of angiogenic activity of human endometrial endothelial cells in culture by ovarian steroids.* J Clin Endocrinol Metab, 2004. **89**(11): p. 5794-802.
22. Morales, D.E., et al., *Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model.* Circulation, 1995. **91**(3): p. 755-63.
23. Razandi, M., A. Pedram, and E.R. Levin, *Estrogen signals to the preservation of endothelial cell form and function.* J Biol Chem, 2000. **275**(49): p. 38540-6.
24. Ross, R., *The pathogenesis of atherosclerosis: a perspective for the 1990s.* Nature, 1993. **362**(6423): p. 801-9.
25. Espinosa, E., B.S. Oemar, and T.F. Luscher, *17 beta-Estradiol and smooth muscle cell proliferation in aortic cells of male and female rats.* Biochem Biophys Res Commun, 1996. **221**(1): p. 8-14.
26. Kolodgie, F.D., et al., *Estradiol attenuates directed migration of vascular smooth muscle cells in vitro.* Am J Pathol, 1996. **148**(3): p. 969-76.
27. Sunday, L., et al., *Estrogen and progestagens differentially modulate vascular proinflammatory factors.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E261-7.
28. Ospina, J.A., et al., *Estrogen suppresses IL-1beta-mediated induction of COX-2 pathway in rat cerebral blood vessels.* Am J Physiol Heart Circ Physiol, 2004. **286**(5): p. H2010-9.
29. Beyer, C., *Estrogen and the developing mammalian brain.* Anat Embryol (Berl), 1999. **199**(5): p. 379-90.
30. Madeira, M.D. and A.R. Lieberman, *Sexual dimorphism in the mammalian limbic system.* Prog Neurobiol, 1995. **45**(4): p. 275-333.
31. Arnold, A.P. and S.M. Breedlove, *Organizational and activational effects of sex steroids on brain and behavior: a reanalysis.* Horm Behav, 1985. **19**(4): p. 469-98.
32. Arnold, A.P. and R.A. Gorski, *Gonadal steroid induction of structural sex differences in the central nervous system.* Annu Rev Neurosci, 1984. **7**: p. 413-42.
33. Handa, R.J., et al., *Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis.* Horm Behav, 1994. **28**(4): p. 464-76.
34. McCormick, C.M., et al., *Neonatal sex hormones have 'organizational' effects on the hypothalamic-pituitary-adrenal axis of male rats.* Brain Res Dev Brain Res, 1998. **105**(2): p. 295-307.
35. Critchlow, V., et al., *Sex difference in resting pituitary-adrenal function in the rat.* Am J Physiol, 1963. **205**(5): p. 807-15.
36. Pfaff, D.W. and Y. Sakuma, *Facilitation of the lordosis reflex of female rats from the ventromedial nucleus of the hypothalamus.* J Physiol, 1979. **288**: p. 189-202.
37. Ogawa, S., et al., *Reversal of sex roles in genetic female mice by disruption of estrogen receptor gene.* Neuroendocrinology, 1996. **64**(6): p. 467-70.
38. Calizo, L.H. and L.M. Flanagan-Cato, *Estrogen selectively regulates spine density within the dendritic arbor of rat ventromedial hypothalamic neurons.* J Neurosci, 2000. **20**(4): p. 1589-96.
39. Cooke, B.M. and C.S. Woolley, *Gonadal hormone modulation of dendrites in the mammalian CNS.* J Neurobiol, 2005. **64**(1): p. 34-46.

40. Pfaff, D., *Hormone-driven mechanisms in the central nervous system facilitate the analysis of mammalian behaviours.* J Endocrinol, 2005. **184**(3): p. 447-53.
41. Toran-Allerand, C.D., *Mechanisms of estrogen action during neural development: mediation by interactions with the neurotrophins and their receptors?* J Steroid Biochem Mol Biol, 1996. **56**(1-6 Spec No): p. 169-78.
42. Ferreira, A. and A. Caceres, *Estrogen-enhanced neurite growth: evidence for a selective induction of Tau and stable microtubules.* J Neurosci, 1991. **11**(2): p. 392-400.
43. Wise, P.M., et al., *Estrogens: trophic and protective factors in the adult brain.* Front Neuroendocrinol, 2001. **22**(1): p. 33-66.
44. Luine, V.N., et al., *Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters.* Horm Behav, 1998. **34**(2): p. 149-62.
45. Daniel, J.M., et al., *Estrogen enhances performance of female rats during acquisition of a radial arm maze.* Horm Behav, 1997. **32**(3): p. 217-25.
46. Gould, E., et al., *Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood.* J Neurosci, 1990. **10**(4): p. 1286-91.
47. McEwen, B.S., P. Tanapat, and N.G. Weiland, *Inhibition of dendritic spine induction on hippocampal CA1 pyramidal neurons by a nonsteroidal estrogen antagonist in female rats.* Endocrinology, 1999. **140**(3): p. 1044-7.
48. Paganini-Hill, A., *Estrogen replacement therapy and stroke.* Prog Cardiovasc Dis, 1995. **38**(3): p. 223-42.
49. Henderson, V.W., et al., *Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects.* Arch Neurol, 1994. **51**(9): p. 896-900.
50. Tang, M.X., et al., *Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease.* Lancet, 1996. **348**(9025): p. 429-32.
51. Dubal, D.B., et al., *Estradiol modulates bcl-2 in cerebral ischemia: a potential role for estrogen receptors.* J Neurosci, 1999. **19**(15): p. 6385-93.
52. Dubal, D.B., et al., *Estradiol protects against ischemic injury.* J Cereb Blood Flow Metab, 1998. **18**(11): p. 1253-8.
53. Miller, D.B., et al., *The impact of gender and estrogen on striatal dopaminergic neurotoxicity.* Ann N Y Acad Sci, 1998. **844**: p. 153-65.
54. Honda, K., et al., *Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons.* J Neurosci Res, 2000. **60**(3): p. 321-7.
55. Singer, C.A., et al., *The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons.* J Neurosci, 1999. **19**(7): p. 2455-63.
56. Mize, A.L., R.A. Shapiro, and D.M. Dorsa, *Estrogen receptor-mediated neuroprotection from oxidative stress requires activation of the mitogen-activated protein kinase pathway.* Endocrinology, 2003. **144**(1): p. 306-12.
57. Nilsen, J. and R. Diaz Brinton, *Mechanism of estrogen-mediated neuroprotection: regulation of mitochondrial calcium and Bcl-2 expression.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2842-7.
58. Wu, T.W., et al., *17Beta-estradiol induced Ca2+ influx via L-type calcium channels activates the Src/ERK/cyclic-AMP response element binding protein*

signal pathway and BCL-2 expression in rat hippocampal neurons: a potential initiation mechanism for estrogen-induced neuroprotection. Neuroscience, 2005. **135**(1): p. 59-72.

59. Kelly, M.J., J. Qiu, and O.K. Ronnekleiv, *Estrogen signaling in the hypothalamus.* Vitam Horm, 2005. **71**: p. 123-45.
60. Liang, Y.Q., et al., *Estrogen receptor beta is involved in the anorectic action of estrogen.* Int J Obes Relat Metab Disord, 2002. **26**(8): p. 1103-9.
61. Bachmann, G.A., *Vasomotor flushes in menopausal women.* Am J Obstet Gynecol, 1999. **180**(3 Pt 2): p. S312-6.
62. Freedman, R.R. and C.M. Blacker, *Estrogen raises the sweating threshold in postmenopausal women with hot flashes.* Fertil Steril, 2002. **77**(3): p. 487-90.
63. Wyss, J.M. and S.H. Carlson, *Effects of hormone replacement therapy on the sympathetic nervous system and blood pressure.* Curr Hypertens Rep, 2003. **5**(3): p. 241-6.
64. Blum, I., et al., *The effect of estrogen replacement therapy on plasma serotonin and catecholamines of postmenopausal women.* Isr J Med Sci, 1996. **32**(12): p. 1158-62.
65. Rosano, G.M., et al., *Effect of estrogen replacement therapy on heart rate variability and heart rate in healthy postmenopausal women.* Am J Cardiol, 1997. **80**(6): p. 815-7.
66. el-Mas, M.M. and A.A. Abdel-Rahman, *Estrogen enhances baroreflex control of heart rate in conscious ovariectomized rats.* Can J Physiol Pharmacol, 1998. **76**(4): p. 381-6.
67. Vongpatanasin, W., et al., *Transdermal estrogen replacement therapy decreases sympathetic activity in postmenopausal women.* Circulation, 2001. **103**(24): p. 2903-8.
68. Liu, C.C., T.B. Kuo, and C.C. Yang, *Effects of estrogen on gender-related autonomic differences in humans.* Am J Physiol Heart Circ Physiol, 2003. **285**(5): p. H2188-93.
69. De Meersman, R.E., et al., *Estrogen replacement, vascular distensibility, and blood pressures in postmenopausal women.* Am J Physiol, 1998. **274**(5 Pt 2): p. H1539-44.
70. Hunt, B.E., et al., *Estrogen replacement therapy improves baroreflex regulation of vascular sympathetic outflow in postmenopausal women.* Circulation, 2001. **103**(24): p. 2909-14.
71. Reckelhoff, J.F., *Gender differences in the regulation of blood pressure.* Hypertension, 2001. **37**(5): p. 1199-208.
72. Reckelhoff, J.F., H. Zhang, and J.P. Granger, *Testosterone exacerbates hypertension and reduces pressure-natriuresis in male spontaneously hypertensive rats.* Hypertension, 1998. **31**(1 Pt 2): p. 435-9.
73. Ashton, N. and R.J. Balment, *Sexual dimorphism in renal function and hormonal status of New Zealand genetically hypertensive rats.* Acta Endocrinol (Copenh), 1991. **124**(1): p. 91-7.
74. Crofton, J.T., M. Ota, and L. Share, *Role of vasopressin, the renin-angiotensin system and sex in Dahl salt-sensitive hypertension.* J Hypertens, 1993. **11**(10): p. 1031-8.

75. He, X.R., et al., *Effects of 17beta-estradiol on sympathetic activity and pressor response to phenylephrine in ovariectomized rats.* Am J Physiol, 1998. **275**(4 Pt 2): p. R1202-8.
76. Saleh, T.M. and B.J. Connell, *17beta-estradiol modulates baroreflex sensitivity and autonomic tone of female rats.* J Auton Nerv Syst, 2000. **80**(3): p. 148-61.
77. Mohamed, M.K., M.M. El-Mas, and A.A. Abdel-Rahman, *Estrogen enhancement of baroreflex sensitivity is centrally mediated.* Am J Physiol, 1999. **276**(4 Pt 2): p. R1030-7.
78. Saleh, M.C., B.J. Connell, and T.M. Saleh, *Medullary and intrathecal injections of 17beta-estradiol in male rats.* Brain Res, 2000. **867**(1-2): p. 200-9.
79. Saleh, M.C., B.J. Connell, and T.M. Saleh, *Autonomic and cardiovascular reflex responses to central estrogen injection in ovariectomized female rats.* Brain Res, 2000. **879**(1-2): p. 105-14.
80. Gerrits, M., et al., *Cyclic estradiol replacement attenuates stress-induced c-Fos expression in the PVN of ovariectomized rats.* Brain Res Bull, 2005. **67**(1-2): p. 147-55.
81. Ueyama, T., et al., *Estrogen alters c-Fos response to immobilization stress in the brain of ovariectomized rats.* Brain Res, 2006. **1084**(1): p. 67-79.
82. Dayas, C.V., et al., *Effects of chronic oestrogen replacement on stress-induced activation of hypothalamic-pituitary-adrenal axis control pathways.* J Neuroendocrinol, 2000. **12**(8): p. 784-94.
83. McCubbin, J.A., et al., *Blood pressure control and hormone replacement therapy in postmenopausal women at risk for coronary heart disease.* Am Heart J, 2002. **143**(4): p. 711-7.
84. Sung, B.H., et al., *Estrogen improves abnormal norepinephrine-induced vasoconstriction in postmenopausal women.* J Hypertens, 1999. **17**(4): p. 523-8.
85. Komesaroff, P.A., M.D. Esler, and K. Sudhir, *Estrogen supplementation attenuates glucocorticoid and catecholamine responses to mental stress in perimenopausal women.* J Clin Endocrinol Metab, 1999. **84**(2): p. 606-10.
86. Cherney, A., H. Edgell, and T.L. Krukoff, *NO mediates effects of estrogen on central regulation of blood pressure in restrained, ovariectomized rats.* Am J Physiol Regul Integr Comp Physiol, 2003. **285**(4): p. R842-9.
87. Morimoto, K., et al., *Estrogen replacement suppresses stress-induced cardiovascular responses in ovariectomized rats.* Am J Physiol Heart Circ Physiol, 2004. **287**(5): p. H1950-6.
88. Serova, L.I., S. Maharjan, and E.L. Sabban, *Estrogen modifies stress response of catecholamine biosynthetic enzyme genes and cardiovascular system in ovariectomized female rats.* Neuroscience, 2005. **132**(2): p. 249-59.
89. Burgess, L.H. and R.J. Handa, *Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats.* Endocrinology, 1992. **131**(3): p. 1261-9.
90. Lund, T.D., L.R. Hinds, and R.J. Handa, *The androgen 5alpha-dihydrotestosterone and its metabolite 5alpha-androstan-3beta, 17beta-diol inhibit the hypothalamo-pituitary-adrenal response to stress by acting through estrogen receptor beta-expressing neurons in the hypothalamus.* J Neurosci, 2006. **26**(5): p. 1448-56.

91. Greene, G.L., et al., *Sequence and expression of human estrogen receptor complementary DNA.* Science, 1986. **231**(4742): p. 1150-4.
92. Green, S., et al., *Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A.* Nature, 1986. **320**(6058): p. 134-9.
93. Kuiper, G.G., et al., *Cloning of a novel receptor expressed in rat prostate and ovary.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5925-30.
94. Pettersson, K. and J.A. Gustafsson, *Role of estrogen receptor beta in estrogen action.* Annu Rev Physiol, 2001. **63**: p. 165-92.
95. Koehler, K.F., et al., *Reflections on the discovery and significance of estrogen receptor beta.* Endocr Rev, 2005. **26**(3): p. 465-78.
96. Kuiper, G.G., et al., *The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems.* Front Neuroendocrinol, 1998. **19**(4): p. 253-86.
97. Thomas, P., et al., *Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells.* Endocrinology, 2005. **146**(2): p. 624-32.
98. Prossnitz, E.R., J.B. Arterburn, and L.A. Sklar, *GPR30: A G protein-coupled receptor for estrogen.* Mol Cell Endocrinol, 2007. **265-266**: p. 138-42.
99. Brailoiu, E., et al., *Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system.* J Endocrinol, 2007. **193**(2): p. 311-21.
100. Shughrue, P.J., M.V. Lane, and I. Merchenthaler, *Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system.* J Comp Neurol, 1997. **388**(4): p. 507-25.
101. Gonzalez, M., et al., *Distribution patterns of estrogen receptor alpha and beta in the human cortex and hippocampus during development and adulthood.* J Comp Neurol, 2007. **503**(6): p. 790-802.
102. Matthews, J. and J.A. Gustafsson, *Estrogen signaling: a subtle balance between ER alpha and ER beta.* Mol Interv, 2003. **3**(5): p. 281-92.
103. Nilsson, S., et al., *Mechanisms of estrogen action.* Physiol Rev, 2001. **81**(4): p. 1535-65.
104. Dechering, K., C. Boersma, and S. Mosselman, *Estrogen receptors alpha and beta: two receptors of a kind?* Curr Med Chem, 2000. **7**(5): p. 561-76.
105. Li, R. and Y. Shen, *Estrogen and brain: synthesis, function and diseases.* Front Biosci, 2005. **10**: p. 257-67.
106. Leung, Y.K., et al., *Estrogen receptor (ER)-beta isoforms: a key to understanding ER-beta signaling.* Proc Natl Acad Sci U S A, 2006. **103**(35): p. 13162-7.
107. Filardo, E., et al., *Activation of the Novel Estrogen Receptor G Protein-Coupled Receptor 30 (GPR30) at the Plasma Membrane.* Endocrinology, 2007. **148**(7): p. 3236-45.
108. Hall, J.M., J.F. Couse, and K.S. Korach, *The multifaceted mechanisms of estradiol and estrogen receptor signaling.* J Biol Chem, 2001. **276**(40): p. 36869-72.
109. Luconi, M., G. Forti, and E. Baldi, *Genomic and nongenomic effects of estrogens: molecular mechanisms of action and clinical implications for male reproduction.* J Steroid Biochem Mol Biol, 2002. **80**(4-5): p. 369-81.

110. Toran-Allerand, C.D., M. Singh, and G. Setalo, Jr., *Novel mechanisms of estrogen action in the brain: new players in an old story.* Front Neuroendocrinol, 1999. **20**(2): p. 97-121.
111. Bjornstrom, L. and M. Sjoberg, *Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes.* Mol Endocrinol, 2005. **19**(4): p. 833-42.
112. O'Lone, R., et al., *Genomic targets of nuclear estrogen receptors.* Mol Endocrinol, 2004. **18**(8): p. 1859-75.
113. Kushner, P.J., et al., *Estrogen receptor pathways to AP-1.* J Steroid Biochem Mol Biol, 2000. **74**(5): p. 311-7.
114. Webb, P., et al., *The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions.* Mol Endocrinol, 1999. **13**(10): p. 1672-85.
115. Paech, K., et al., *Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites.* Science, 1997. **277**(5331): p. 1508-10.
116. Duan, R., W. Porter, and S. Safe, *Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation.* Endocrinology, 1998. **139**(4): p. 1981-90.
117. Porter, W., et al., *Functional synergy between the transcription factor Sp1 and the estrogen receptor.* Mol Endocrinol, 1997. **11**(11): p. 1569-80.
118. Kelly, M.J. and E.R. Levin, *Rapid actions of plasma membrane estrogen receptors.* Trends Endocrinol Metab, 2001. **12**(4): p. 152-6.
119. Improta-Brears, T., et al., *Estrogen-induced activation of mitogen-activated protein kinase requires mobilization of intracellular calcium.* Proc Natl Acad Sci U S A, 1999. **96**(8): p. 4686-91.
120. Farhat, M.Y., et al., *Estradiol increases cyclic adenosine monophosphate in rat pulmonary vascular smooth muscle cells by a nongenomic mechanism.* J Pharmacol Exp Ther, 1996. **276**(2): p. 652-7.
121. Xia, Y. and T.L. Krukoff, *Estrogen induces nitric oxide production via activation of constitutive nitric oxide synthases in human neuroblastoma cells.* Endocrinology, 2004. **145**(10): p. 4550-7.
122. Stirone, C., et al., *Estrogen receptor activation of phosphoinositide-3 kinase, akt, and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects.* Mol Pharmacol, 2005. **67**(1): p. 105-13.
123. Haynes, M.P., et al., *Src kinase mediates phosphatidylinositol 3-kinase/Akt-dependent rapid endothelial nitric-oxide synthase activation by estrogen.* J Biol Chem, 2003. **278**(4): p. 2118-23.
124. Florian, M., et al., *Estrogen induced changes in Akt-dependent activation of endothelial nitric oxide synthase and vasodilation.* Steroids, 2004. **69**(10): p. 637-45.
125. Razandi, M., et al., *Plasma membrane estrogen receptors exist and functions as dimers.* Mol Endocrinol, 2004. **18**(12): p. 2854-65.
126. Milner, T.A., et al., *Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation.* J Comp Neurol, 2005. **491**(2): p. 81-95.

127. Chambliss, K.L., et al., *ERbeta has nongenomic action in caveolae.* Mol Endocrinol, 2002. **16**(5): p. 938-46.

128. Acconcia, F., et al., *Palmitoylation-dependent estrogen receptor alpha membrane localization: regulation by 17beta-estradiol.* Mol Biol Cell, 2005. **16**(1): p. 231-7.

129. Pedram, A., et al., *A conserved mechanism for steroid receptor translocation to the plasma membrane.* J Biol Chem, 2007. **282**(31): p. 22278-88.

130. Galluzzo, P., et al., *Role of ERbeta palmitoylation in the inhibition of human colon cancer cell proliferation.* Endocr Relat Cancer, 2007. **14**(1): p. 153-67.

131. Revankar, C.M., et al., *A transmembrane intracellular estrogen receptor mediates rapid cell signaling.* Science, 2005. **307**(5715): p. 1625-30.

132. Filardo, E.J., et al., *Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis.* Mol Endocrinol, 2002. **16**(1): p. 70-84.

133. Funakoshi, T., et al., *G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane.* Biochem Biophys Res Commun, 2006. **346**(3): p. 904-10.

134. Hewitt, S.C., B.J. Deroo, and K.S. Korach, *Signal transduction. A new mediator for an old hormone?* Science, 2005. **307**(5715): p. 1572-3.

135. Marino, M., et al., *Distinct nongenomic signal transduction pathways controlled by 17beta-estradiol regulate DNA synthesis and cyclin D(1) gene transcription in HepG2 cells.* Mol Biol Cell, 2002. **13**(10): p. 3720-9.

136. Kousteni, S., et al., *Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids.* J Clin Invest, 2003. **111**(11): p. 1651-64.

137. Kawagoe, J., et al., *Raloxifene inhibits estrogen-induced up-regulation of telomerase activity in a human breast cancer cell line.* J Biol Chem, 2003. **278**(44): p. 43363-72.

138. Vasudevan, N., L.M. Kow, and D. Pfaff, *Integration of steroid hormone initiated membrane action to genomic function in the brain.* Steroids, 2005. **70**(5-7): p. 388-96.

139. Lannigan, D.A., *Estrogen receptor phosphorylation.* Steroids, 2003. **68**(1): p. 1-9.

140. Singh, M., et al., *Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways.* J Neurosci, 1999. **19**(4): p. 1179-88.

141. Ivanova, T., et al., *Rapid stimulation of the PI3-kinase/Akt signalling pathway in developing midbrain neurones by oestrogen.* J Neuroendocrinol, 2002. **14**(1): p. 73-9.

142. Akama, K.T. and B.S. McEwen, *Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway.* J Neurosci, 2003. **23**(6): p. 2333-9.

143. Mannella, P. and R.D. Brinton, *Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action.* J Neurosci, 2006. **26**(37): p. 9439-47.

144. Jover-Mengual, T., R.S. Zukin, and A.M. Etgen, *MAPK signaling is critical to estradiol protection of CA1 neurons in global ischemia.* Endocrinology, 2007. **148**(3): p. 1131-43.
145. Fitzpatrick, J.L., et al., *Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway.* J Neurochem, 2002. **82**(3): p. 674-82.
146. Numakawa, Y., et al., *17beta-estradiol protects cortical neurons against oxidative stress-induced cell death through reduction in the activity of mitogen-activated protein kinase and in the accumulation of intracellular calcium.* Endocrinology, 2007. **148**(2): p. 627-37.
147. Wang, R., et al., *Inhibition of MLK3-MKK4/7-JNK1/2 pathway by Akt1 in exogenous estrogen-induced neuroprotection against transient global cerebral ischemia by a non-genomic mechanism in male rats.* J Neurochem, 2006. **99**(6): p. 1543-54.
148. Simoncini, T., et al., *Genomic and non-genomic effects of estrogens on endothelial cells.* Steroids, 2004. **69**(8-9): p. 537-42.
149. Simoncini, T., P. Mannella, and A.R. Genazzani, *Rapid estrogen actions in the cardiovascular system.* Ann N Y Acad Sci, 2006. **1089**: p. 424-30.
150. Krukoff, T.L., *Central actions of nitric oxide in regulation of autonomic functions.* Brain Res Brain Res Rev, 1999. **30**(1): p. 52-65.
151. Guix, F.X., et al., *The physiology and pathophysiology of nitric oxide in the brain.* Prog Neurobiol, 2005. **76**(2): p. 126-52.
152. Stern, J.E., *Nitric oxide and homeostatic control: an intercellular signalling molecule contributing to autonomic and neuroendocrine integration?* Prog Biophys Mol Biol, 2004. **84**(2-3): p. 197-215.
153. Mungrue, I.N., et al., *From molecules to mammals: what's NOS got to do with it?* Acta Physiol Scand, 2003. **179**(2): p. 123-35.
154. Lin, L.H., O. Taktakishvili, and W.T. Talman, *Identification and localization of cell types that express endothelial and neuronal nitric oxide synthase in the rat nucleus tractus solitarii.* Brain Res, 2007. **1171**: p. 42-51.
155. Oka, M., et al., *Functional expression of constitutive nitric oxide synthases regulated by voltage-gated Na+ and Ca2+ channels in cultured human astrocytes.* Glia, 2004. **46**(1): p. 53-62.
156. Kone, B.C., et al., *Protein interactions with nitric oxide synthases: controlling the right time, the right place, and the right amount of nitric oxide.* Am J Physiol Renal Physiol, 2003. **285**(2): p. F178-90.
157. Alderton, W.K., C.E. Cooper, and R.G. Knowles, *Nitric oxide synthases: structure, function and inhibition.* Biochem J, 2001. **357**(Pt 3): p. 593-615.
158. Fleming, I. and R. Busse, *Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase.* Am J Physiol Regul Integr Comp Physiol, 2003. **284**(1): p. R1-12.
159. Rameau, G.A., et al., *Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AMPA receptor trafficking and neuronal cell death.* J Neurosci, 2007. **27**(13): p. 3445-55.
160. Zhang, J. and S.H. Snyder, *Nitric oxide in the nervous system.* Annu Rev Pharmacol Toxicol, 1995. **35**: p. 213-33.

161. Bains, J.S. and A.V. Ferguson, *Nitric oxide depolarizes type II paraventricular nucleus neurons in vitro.* Neuroscience, 1997. **79**(1): p. 149-59.
162. Travagli, R.A. and R.A. Gillis, *Nitric oxide-mediated excitatory effect on neurons of dorsal motor nucleus of vagus.* Am J Physiol, 1994. **266**(1 Pt 1): p. G154-60.
163. Malen, P.L. and P.F. Chapman, *Nitric oxide facilitates long-term potentiation, but not long-term depression.* J Neurosci, 1997. **17**(7): p. 2645-51.
164. Schuman, E.M. and D.V. Madison, *A requirement for the intercellular messenger nitric oxide in long-term potentiation.* Science, 1991. **254**(5037): p. 1503-6.
165. Mon[illegible]gue, P.R., et al., *Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex.* Science, 1994. **263**(5149): p. 973-7.
166. Hanbauer, I., et al., *Role of nitric oxide in NMDA-evoked release of [3H]-dopamine from striatal slices.* Neuroreport, 1992. **3**(5): p. 409-12.
167. Prast, H. and A. Philippu, *Nitric oxide releases acetylcholine in the basal forebrain.* Eur J Pharmacol, 1992. **216**(1): p. 139-40.
168. Vincent, S.R. and H. Kimura, *Histochemical mapping of nitric oxide synthase in the rat brain.* Neuroscience, 1992. **46**(4): p. 755-84.
169. Kantzides, A. and E. Badoer, *nNOS-containing neurons in the hypothalamus and medulla project to the RVLM.* Brain Res, 2005. **1037**(1-2): p. 25-34.
170. Cunha, R.S., A.M. Cabral, and E.C. Vasquez, *Evidence that the autonomic nervous system plays a major role in the L-NAME-induced hypertension in conscious rats.* Am J Hypertens, 1993. **6**(9): p. 806-9.
171. Nurminen, M.L. and H. Vapaatalo, *Effect of intracerebroventricular and intravenous administration of nitric oxide donors on blood pressure and heart rate in anaesthetized rats.* Br J Pharmacol, 1996. **119**(7): p. 1422-6.
172. Rees, D.D., et al., *Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo.* Br J Pharmacol, 1990. **101**(3): p. 746-52.
173. Nurminen, M.L., A. Ylikorkala, and H. Vapaatalo, *Central inhibition of nitric oxide synthesis increases blood pressure and heart rate in anesthetized rats.* Methods Find Exp Clin Pharmacol, 1997. **19**(1): p. 35-41.
174. Nishimura, M., et al., *Cardiovascular regulation by L-arginine in the brain of rats: role of the brain renin-angiotensin system and nitric oxide.* Am J Hypertens, 1997. **10**(4 Pt 1): p. 389-96.
175. Zhang, K. and K.P. Patel, *Effect of nitric oxide within the paraventricular nucleus on renal sympathetic nerve discharge: role of GABA.* Am J Physiol, 1998. **275**(3 Pt 2): p. R728-34.
176. Krukoff, T.L. and P. Khalili, *Stress-induced activation of nitric oxide-producing neurons in the rat brain.* J Comp Neurol, 1997. **377**(4): p. 509-19.
177. Shirakawa, T., et al., *Immobilization induces acute nitric oxide production in the rat hypothalamus: a role of ionotropic glutamate receptors in the paraventricular nucleus.* Endocrinology, 2004. **145**(8): p. 3603-7.
178. McNeill, A.M., et al., *Chronic estrogen treatment increases levels of endothelial nitric oxide synthase protein in rat cerebral microvessels.* Stroke, 1999. **30**(10): p. 2186-90.
179. Yang, S., L. Bae, and L. Zhang, *Estrogen increases eNOS and NOx release in human coronary artery endothelium.* J Cardiovasc Pharmacol, 2000. **36**(2): p. 242-7.

180. Pelligrino, D.A., et al., *Nitric-oxide-dependent pial arteriolar dilation in the female rat: effects of chronic estrogen depletion and repletion.* Biochem Biophys Res Commun, 2000. **269**(1): p. 165-71.
181. MacRitchie, A.N., et al., *Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium.* Circ Res, 1997. **81**(3): p. 355-62.
182. Govers, R. and T.J. Rabelink, *Cellular regulation of endothelial nitric oxide synthase.* Am J Physiol Renal Physiol, 2001. **280**(2): p. F193-206.
183. Chen, D.B., et al., *Membrane estrogen receptor-dependent extracellular signal-regulated kinase pathway mediates acute activation of endothelial nitric oxide synthase by estrogen in uterine artery endothelial cells.* Endocrinology, 2004. **145**(1): p. 113-25.
184. Chambliss, K.L. and P.W. Shaul, *Rapid activation of endothelial NO synthase by estrogen: evidence for a steroid receptor fast-action complex (SRFC) in caveolae.* Steroids, 2002. **67**(6): p. 413-9.
185. Haynes, M.P., et al., *Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells.* Circ Res, 2000. **87**(8): p. 677-82.
186. Stefano, G.B., et al., *Cell-surface estrogen receptors mediate calcium-dependent nitric oxide release in human endothelia.* Circulation, 2000. **101**(13): p. 1594-7.
187. Klinge, C.M., et al., *Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells.* J Biol Chem, 2005. **280**(9): p. 7460-8.
188. Simoncini, T., et al., *Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase.* Nature, 2000. **407**(6803): p. 538-41.
189. Mendelsohn, M.E., *Mechanisms of estrogen action in the cardiovascular system.* J Steroid Biochem Mol Biol, 2000. **74**(5): p. 337-43.
190. Chambliss, K.L., et al., *Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae.* Circ Res, 2000. **87**(11): p. E44-52.
191. Jesmin, S., et al., *Estrogen deprivation and replacement modulate cerebral capillary density with vascular expression of angiogenic molecules in middle-aged female rats.* J Cereb Blood Flow Metab, 2003. **23**(2): p. 181-9.
192. Haas, E., et al., *Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis.* Hypertension, 2007. **49**(6): p. 1358-63.
193. Okamura, H., M. Yokosuka, and S. Hayashi, *Estrogenic induction of NADPH-diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in the female rat.* J Neuroendocrinol, 1994. **6**(6): p. 597-601.
194. Okamura, H., et al., *Colocalization of NADPH-diaphorase and estrogen receptor immunoreactivity in the rat ventromedial hypothalamic nucleus: stimulatory effect of estrogen on NADPH-diaphorase activity.* Endocrinology, 1994. **135**(4): p. 1705-8.
195. Sanchez, F., et al., *Reduced nicotinamide adenine dinucleotide phosphate-diaphorase activity in the paraventricular nucleus of the rat hypothalamus is modulated by estradiol.* Neurosci Lett, 1998. **253**(2): p. 75-8.

196. Ceccatelli, S., et al., *Estradiol regulation of nitric oxide synthase mRNAs in rat hypothalamus.* Neuroendocrinology, 1996. **64**(5): p. 357-63.
197. Grohe, C., et al., *17 Beta-estradiol regulates nNOS and eNOS activity in the hippocampus.* Neuroreport, 2004. **15**(1): p. 89-93.
198. d'Anglemont de Tassigny, X., et al., *Coupling of neuronal nitric oxide synthase to NMDA receptors via postsynaptic density-95 depends on estrogen and contributes to the central control of adult female reproduction.* J Neurosci, 2007. **27**(23): p. 6103-14.
199. Knauf, C., et al., *Evidence for a spontaneous nitric oxide release from the rat median eminence: influence on gonadotropin-releasing hormone release.* Endocrinology, 2001. **142**(6): p. 2343-50.
200. Wen, Y., et al., *nNOS is involved in estrogen mediated neuroprotection in neuroblastoma cells.* Neuroreport, 2004. **15**(9): p. 1515-8.
201. Prevot, V., et al., *Estradiol coupling to endothelial nitric oxide stimulates gonadotropin-releasing hormone release from rat median eminence via a membrane receptor.* Endocrinology, 1999. **140**(2): p. 652-9.
202. McNeill, A.M., et al., *Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone.* Stroke, 2002. **33**(6): p. 1685-91.
203. Xu, H.L., et al., *The key role of caveolin-1 in estrogen-mediated regulation of endothelial nitric oxide synthase function in cerebral arterioles in vivo.* J Cereb Blood Flow Metab, 2001. **21**(8): p. 907-13.
204. Knauf, C., et al., *Variation of endothelial nitric oxide synthase synthesis in the median eminence during the rat estrous cycle: an additional argument for the implication of vascular blood vessel in the control of GnRH release.* Endocrinology, 2001. **142**(10): p. 4288-94.
205. Lee, S.Y., et al., *17beta-estradiol activates ICI 182,780-sensitive estrogen receptors and cyclic GMP-dependent thioredoxin expression for neuroprotection.* Faseb J, 2003. **17**(8): p. 947-8.
206. Audesirk, T., et al., *Enhancement of dendritic branching in cultured hippocampal neurons by 17beta-estradiol is mediated by nitric oxide.* Int J Dev Neurosci, 2003. **21**(4): p. 225-33.
207. Patisaul, H.B., et al., *Genistein affects ER beta- but not ER alpha-dependent gene expression in the hypothalamus.* Endocrinology, 2002. **143**(6): p. 2189-97.
208. Somponpun, S. and C.D. Sladek, *Role of estrogen receptor-beta in regulation of vasopressin and oxytocin release in vitro.* Endocrinology, 2002. **143**(8): p. 2899-904.
209. Harrington, W.R., et al., *Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression.* Mol Cell Endocrinol, 2003. **206**(1-2): p. 13-22.
210. Stauffer, S.R., et al., *Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists.* J Med Chem, 2000. **43**(26): p. 4934-47.
211. Sun, J., et al., *Antagonists selective for estrogen receptor alpha.* Endocrinology, 2002. **143**(3): p. 941-7.

212. Xu, Y. and T.L. Krukoff, *Decrease in arterial pressure induced by adrenomedullin in the hypothalamic paraventricular nucleus is mediated by nitric oxide and GABA.* Regul Pept, 2004. **119**(1-2): p. 21-30.
213. House, S.B., et al., *Stationary organotypic cultures of oxytocin and vasopressin magnocellular neurones from rat and mouse hypothalamus.* J Neuroendocrinol, 1998. **10**(11): p. 849-61.
214. Moser, K.V., et al., *Brain capillaries and cholinergic neurons persist in organotypic brain slices in the absence of blood flow.* Eur J Neurosci, 2003. **18**(1): p. 85-94.
215. Veltkamp, R., et al., *Transient focal ischemia increases endothelial nitric oxide synthase in cerebral blood vessels.* Stroke, 2002. **33**(11): p. 2704-10.
216. Kiss, J.Z., *Dynamism of chemoarchitecture in the hypothalamic paraventricular nucleus.* Brain Res Bull, 1988. **20**(6): p. 699-708.
217. Laflamme, N., et al., *Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype.* J Neurobiol, 1998. **36**(3): p. 357-78.
218. Xu, Y. and T.L. Krukoff, *Adrenomedullin Stimulates Nitric Oxide Production from Primary Rat Hypothalamic Neurons: Roles of Calcium and Phosphatases.* Mol Pharmacol, 2007.
219. Jiang, X.Y., et al., *Methods for isolating highly-enriched embryonic spinal cord neurons: a comparison between enzymatic and mechanical dissociations.* J Neurosci Methods, 2006. **158**(1): p. 13-8.
220. Gingerich, S. and T.L. Krukoff, *Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an estrogen receptor beta-dependent mechanism in hypothalamic slice cultures.* Endocrinology, 2005. **146**(7): p. 2933-41.
221. Esslimani-Sahla, M., et al., *Increased estrogen receptor betacx expression during mammary carcinogenesis.* Clin Cancer Res, 2005. **11**(9): p. 3170-4.
222. Roger, P., et al., *Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors.* Cancer Res, 2001. **61**(6): p. 2537-41.
223. Rossouw, J.E., et al., *Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial.* Jama, 2002. **288**(3): p. 321-33.
224. McDonough, P.G., *The randomized world is not without its imperfections: reflections on the Women's Health Initiative Study.* Fertil Steril, 2002. **78**(5): p. 951-6.
225. Del Rio, G., et al., *Acute estradiol and progesterone administration reduced cardiovascular and catecholamine responses to mental stress in menopausal women.* Neuroendocrinology, 1998. **67**(4): p. 269-74.
226. Russell, K.S., et al., *Estrogen stimulates heat shock protein 90 binding to endothelial nitric oxide synthase in human vascular endothelial cells. Effects on calcium sensitivity and NO release.* J Biol Chem, 2000. **275**(7): p. 5026-30.
227. Hishikawa, K., et al., *Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells.* FEBS Lett, 1995. **360**(3): p. 291-3.

228. Wang, H. and J.F. Morris, *Effects of oestrogen upon nitric oxide synthase NADPH-diaphorase activity in the hypothalamo-neurohypophysial system of the rat.* Neuroscience, 1999. **88**(1): p. 151-8.

229. Kim, J.M., et al., *Ethyl acetate soluble fraction of Cnidium officinale MAKINO inhibits neuronal cell death by reduction of excessive nitric oxide production in lipopolysaccharide-treated rat hippocampal slice cultures and microglia cells.* J Pharmacol Sci, 2003. **92**(1): p. 74-8.

230. Vincent, V.A., S.P. Selwood, and G.M. Murphy, Jr., *Proinflammatory effects of M-CSF and A beta in hippocampal organotypic cultures.* Neurobiol Aging, 2002. **23**(3): p. 349-62.

231. Vutskits, L., E. Gascon, and J.Z. Kiss, *Removal of PSA from NCAM affects the survival of magnocellular vasopressin- and oxytocin-producing neurons in organotypic cultures of the paraventricular nucleus.* Eur J Neurosci, 2003. **17**(10): p. 2119-26.

232. Bayer, L., et al., *Survival of rat MCH (melanin-concentrating hormone) neurons in hypothalamus slice culture: histological, pharmacological and molecular studies.* Cell Tissue Res, 1999. **297**(1): p. 23-33.

233. Perez, S.E., E.Y. Chen, and E.J. Mufson, *Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain.* Brain Res Dev Brain Res, 2003. **145**(1): p. 117-39.

234. Pelligrino, D.A. and E. Galea, *Estrogen and cerebrovascular physiology and pathophysiology.* Jpn J Pharmacol, 2001. **86**(2): p. 137-58.

235. Bianco, N.R., L.J. Chaplin, and M.M. Montano, *Differential induction of quinone reductase by phytoestrogens and protection against estrogen-induced DNA damage.* Biochem J, 2004. **Pt**.

236. Mobley, J.A. and R.W. Brueggemeier, *Estrogen receptor-mediated regulation of oxidative stress and DNA damage in breast cancer.* Carcinogenesis, 2004. **25**(1): p. 3-9.

237. Yoon, H.N., et al., *Effects of estrogen on nitric oxide synthase and histological composition in the rabbit clitoris and vagina.* Int J Impot Res, 2001. **13**(4): p. 205-11.

238. Tostes, R.C., et al., *Effects of estrogen on the vascular system.* Braz J Med Biol Res, 2003. **36**(9): p. 1143-58.

239. McEwen, B., *Estrogen actions throughout the brain.* Recent Prog Horm Res, 2002. **57**: p. 357-84.

240. Segars, J.H. and P.H. Driggers, *Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes.* Trends Endocrinol Metab, 2002. **13**(8): p. 349-54.

241. Dhandapani, K.M. and D.W. Brann, *Protective effects of estrogen and selective estrogen receptor modulators in the brain.* Biol Reprod, 2002. **67**(5): p. 1379-85.

242. Qiu, J., et al., *Rapid signaling of estrogen in hypothalamic neurons involves a novel G-protein-coupled estrogen receptor that activates protein kinase C.* J Neurosci, 2003. **23**(29): p. 9529-40.

243. Beyer, C., J. Pawlak, and M. Karolczak, *Membrane receptors for oestrogen in the brain.* J Neurochem, 2003. **87**(3): p. 545-50.

244. Stefano, G.B., et al., *Estrogen signaling at the cell surface coupled to nitric oxide release in Mytilus edulis nervous system.* Endocrinology, 2003. **144**(4): p. 1234-40.

245. Bektic, J., et al., *Androgen receptor regulation by physiological concentrations of the isoflavonoid genistein in androgen-dependent LNCaP cells is mediated by estrogen receptor beta.* Eur Urol, 2004. **45**(2): p. 245-51; discussion 251.

246. Kuiper, G.G., et al., *Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta.* Endocrinology, 1998. **139**(10): p. 4252-63.

247. Akiyama, T., et al., *Genistein, a specific inhibitor of tyrosine-specific protein kinases.* J Biol Chem, 1987. **262**(12): p. 5592-5.

248. Jonas, J.C., et al., *Multiple effects and stimulation of insulin secretion by the tyrosine kinase inhibitor genistein in normal mouse islets.* Br J Pharmacol, 1995. **114**(4): p. 872-80.

249. Nuedling, S., et al., *Activation of estrogen receptor beta is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes.* FEBS Lett, 2001. **502**(3): p. 103-8.

250. Harris, H.A., J.A. Katzenellenbogen, and B.S. Katzenellenbogen, *Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target tissues in vivo through the use of an ERalpha-selective ligand.* Endocrinology, 2002. **143**(11): p. 4172-7.

251. Geary, G.G., et al., *Selected contribution: cerebrovascular nos and cyclooxygenase are unaffected by estrogen in mice lacking estrogen receptor-alpha.* J Appl Physiol, 2001. **91**(5): p. 2391-9; discussion 2389-90.

252. Samsioe, G., *HRT and cardiovascular disease.* Ann N Y Acad Sci, 2003. **997**: p. 358-72.

253. Rosano, G.M., et al., *Hormone replacement therapy and cardioprotection: the end of the tale?* Ann N Y Acad Sci, 2003. **997**: p. 351-7.

254. Grodstein, F., et al., *A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease.* Ann Intern Med, 2000. **133**(12): p. 933-41.

255. Klaiber, E.L., W. Vogel, and S. Rako, *A critique of the Women's Health Initiative hormone therapy study.* Fertil Steril, 2005. **84**(6): p. 1589-601.

256. Saleh, T.M. and B.J. Connell, *Estrogen-induced autonomic effects are mediated by NMDA and GABAA receptors in the parabrachial nucleus.* Brain Res, 2003. **973**(2): p. 161-70.

257. Swanson, L.W. and P.E. Sawchenko, *Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms.* Neuroendocrinology, 1980. **31**(6): p. 410-7.

258. Ueyama, T., et al., *Estrogen alters c-Fos response to immobilization stress in the brain of ovariectomized rats.* Brain Res, 2006.

259. Isgor, C., et al., *Estrogen receptor beta in the paraventricular nucleus of hypothalamus regulates the neuroendocrine response to stress and is regulated by corticosterone.* Neuroscience, 2003. **121**(4): p. 837-45.

260. Patel, K.P., Y.F. Li, and Y. Hirooka, *Role of nitric oxide in central sympathetic outflow.* Exp Biol Med (Maywood), 2001. **226**(9): p. 814-24.

261. Martin, D.S., T. Segura, and J.R. Haywood, *Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat.* Hypertension, 1991. **18**(1): p. 48-55.
262. Paxinos, G. and C. Watson, *The Rat Brain in Stereotaxic Coordinates.* 1986, Orlando, FL: Academic.
263. Saleh, T.M., B.J. Connell, and A.E. Cribb, *Sympathoexcitatory effects of estrogen in the insular cortex are mediated by GABA.* Brain Res, 2005. **1037**(1-2): p. 114-22.
264. Dampney, R.A., *Functional organization of central pathways regulating the cardiovascular system.* Physiol Rev, 1994. **74**(2): p. 323-64.
265. Lovick, T.A. and J.H. Coote, *Electrophysiological properties of paraventriculo-spinal neurones in the rat.* Brain Res, 1988. **454**(1-2): p. 123-30.
266. Zhang, T.X. and J. Ciriello, *Effect of paraventricular nucleus lesions on arterial pressure and heart rate after aortic baroreceptor denervation in the rat.* Brain Res, 1985. **341**(1): p. 101-9.
267. Kannan, H., Y. Hayashida, and H. Yamashita, *Increase in sympathetic outflow by paraventricular nucleus stimulation in awake rats.* Am J Physiol, 1989. **256**(6 Pt 2): p. R1325-30.
268. Martin, D.S. and J.R. Haywood, *Sympathetic nervous system activation by glutamate injections into the paraventricular nucleus.* Brain Res, 1992. **577**(2): p. 261-7.
269. Krukoff, T.L., et al., *Expression of c-fos protein in rat brain elicited by electrical and chemical stimulation of the hypothalamic paraventricular nucleus.* Neuroendocrinology, 1994. **59**(6): p. 590-602.
270. Hayashi, T., et al., *Biphasic effect of estrogen on neuronal constitutive nitric oxide synthase via Ca(2+)-calmodulin dependent mechanism.* Biochem Biophys Res Commun, 1994. **203**(2): p. 1013-9.
271. Stern, J.E. and W. Zhang, *Preautonomic neurons in the paraventricular nucleus of the hypothalamus contain estrogen receptor beta.* Brain Res, 2003. **975**(1-2): p. 99-109.
272. Kajantie, E. and D.I. Phillips, *The effects of sex and hormonal status on the physiological response to acute psychosocial stress.* Psychoneuroendocrinology, 2006. **31**(2): p. 151-78.
273. Schwartz, A.R., et al., *Toward a causal model of cardiovascular responses to stress and the development of cardiovascular disease.* Psychosom Med, 2003. **65**(1): p. 22-35.
274. Gingerich, S. and T.L. Krukoff, *Estrogen in the paraventricular nucleus attenuates L-glutamate-induced increases in mean arterial pressure through estrogen receptor beta and NO.* Hypertension, 2006. **48**(6): p. 1130-6.
275. Liu, X., et al., *Estrogen provides neuroprotection against activated microglia-induced dopaminergic neuronal injury through both estrogen receptor-alpha and estrogen receptor-beta in microglia.* J Neurosci Res, 2005. **81**(5): p. 653-65.
276. Rune, G.M. and M. Frotscher, *Neurosteroid synthesis in the hippocampus: role in synaptic plasticity.* Neuroscience, 2005. **136**(3): p. 833-42.

277. Escande, A., et al., *Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta.* Biochem Pharmacol, 2006. **71**(10): p. 1459-69.
278. Adak, S., et al., *Neuronal nitric-oxide synthase mutant (Ser-1412 --> Asp) demonstrates surprising connections between heme reduction, NO complex formation, and catalysis.* J Biol Chem, 2001. **276**(2): p. 1244-52.
279. Fulton, D., et al., *Regulation of endothelium-derived nitric oxide production by the protein kinase Akt.* Nature, 1999. **399**(6736): p. 597-601.
280. Song, R.X., Z. Zhang, and R.J. Santen, *Estrogen rapid action via protein complex formation involving ERalpha and Src.* Trends Endocrinol Metab, 2005. **16**(8): p. 347-53.
281. Song, R.X. and R.J. Santen, *Membrane initiated estrogen signaling in breast cancer.* Biol Reprod, 2006. **75**(1): p. 9-16.
282. Migliaccio, A., et al., *Src is an initial target of sex steroid hormone action.* Ann N Y Acad Sci, 2002. **963**: p. 185-90.
283. Belcher, S.M., et al., *Rapid estrogenic regulation of extracellular signal-regulated kinase 1/2 signaling in cerebellar granule cells involves a G protein- and protein kinase A-dependent mechanism and intracellular activation of protein phosphatase 2A.* Endocrinology, 2005. **146**(12): p. 5397-406.
284. Moro, L., et al., *Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase.* Blood, 2005. **105**(1): p. 115-21.
285. Kalita, K., S. Szymczak, and L. Kaczmarek, *Non-nuclear estrogen receptor beta and alpha in the hippocampus of male and female rats.* Hippocampus, 2005. **15**(3): p. 404-12.
286. Nishio, M., Y. Kuroki, and Y. Watanabe, *Subcellular localization of estrogen receptor beta in mouse hippocampus.* Neurosci Lett, 2004. **355**(1-2): p. 109-12.
287. Bu, J., et al., *Glutamate regulates caveolin expression in rat hippocampal neurons.* J Neurosci Res, 2003. **72**(2): p. 185-90.
288. Galbiati, F., et al., *Expression of caveolin-1 and -2 in differentiating PC12 cells and dorsal root ganglion neurons: caveolin-2 is up-regulated in response to cell injury.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 10257-62.
289. Yen, S.H. and J.T. Pan, *Nitric oxide plays an important role in the diurnal change of tuberoinfundibular dopaminergic neuronal activity and prolactin secretion in ovariectomized, estrogen/progesterone-treated rats.* Endocrinology, 1999. **140**(1): p. 286-91.
290. Mastorakos, G., et al., *Pitfalls of the WHIs: Women's Health Initiative.* Ann N Y Acad Sci, 2006. **1092**: p. 331-40.
291. Prentice, R.L., M. Pettinger, and G.L. Anderson, *Statistical issues arising in the Women's Health Initiative.* Biometrics, 2005. **61**(4): p. 899-911; discussion 911-41.
292. MacLennan, A.H., *HRT: a reappraisal of the risks and benefits.* Med J Aust, 2007. **186**(12): p. 643-6.
293. *World development report 1993 - investing in health.* World Bank. 1993, New York: Oxford University Press.

294. Garthwaite, G., et al., *Signaling from blood vessels to CNS axons through nitric oxide.* J Neurosci, 2006. **26**(29): p. 7730-40.

295. Hsieh, Y.C., et al., *G protein-coupled receptor 30-dependent protein kinase A pathway is critical in nongenomic effects of estrogen in attenuating liver injury after trauma-hemorrhage.* Am J Pathol, 2007. **170**(4): p. 1210-8.

296. Kalantaridou, S.N., et al., *Stress and the female reproductive system.* J Reprod Immunol, 2004. **62**(1-2): p. 61-8.

297. Donadio, M.V., et al., *Angiotensin II receptors are upregulated by estradiol and progesterone in the locus coeruleus, median preoptic nucleus and subfornical organ of ovariectomized rats.* Brain Res, 2005. **1065**(1-2): p. 47-52.

298. Retana-Marquez, S., E.D. Salazar, and J. Velazquez-Moctezuma, *Effect of acute and chronic stress on masculine sexual behavior in the rat.* Psychoneuroendocrinology, 1996. **21**(1): p. 39-50.

299. Campagne, D.M., *Should fertilization treatment start with reducing stress?* Hum Reprod, 2006. **21**(7): p. 1651-8.

300. Cwikel, J., Y. Gidron, and E. Sheiner, *Psychological interactions with infertility among women.* Eur J Obstet Gynecol Reprod Biol, 2004. **117**(2): p. 126-31.

301. Beyer, C. and H. Raab, *Nongenomic effects of oestrogen: embryonic mouse midbrain neurones respond with a rapid release of calcium from intracellular stores.* Eur J Neurosci, 1998. **10**(1): p. 255-62.

302. Barletta, F., et al., *Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc.* Mol Endocrinol, 2004. **18**(5): p. 1096-108.

303. Greger, J.G., et al., *Phosphorylation of MNAR promotes estrogen activation of phosphatidylinositol 3-kinase.* Mol Cell Biol, 2007. **27**(5): p. 1904-13.

304. Khan, M.M., et al., *Cloning, expression, and localization of MNAR/PELP1 in rodent brain: colocalization in estrogen receptor-alpha- but not in gonadotropin-releasing hormone-positive neurons.* Endocrinology, 2005. **146**(12): p. 5215-27.